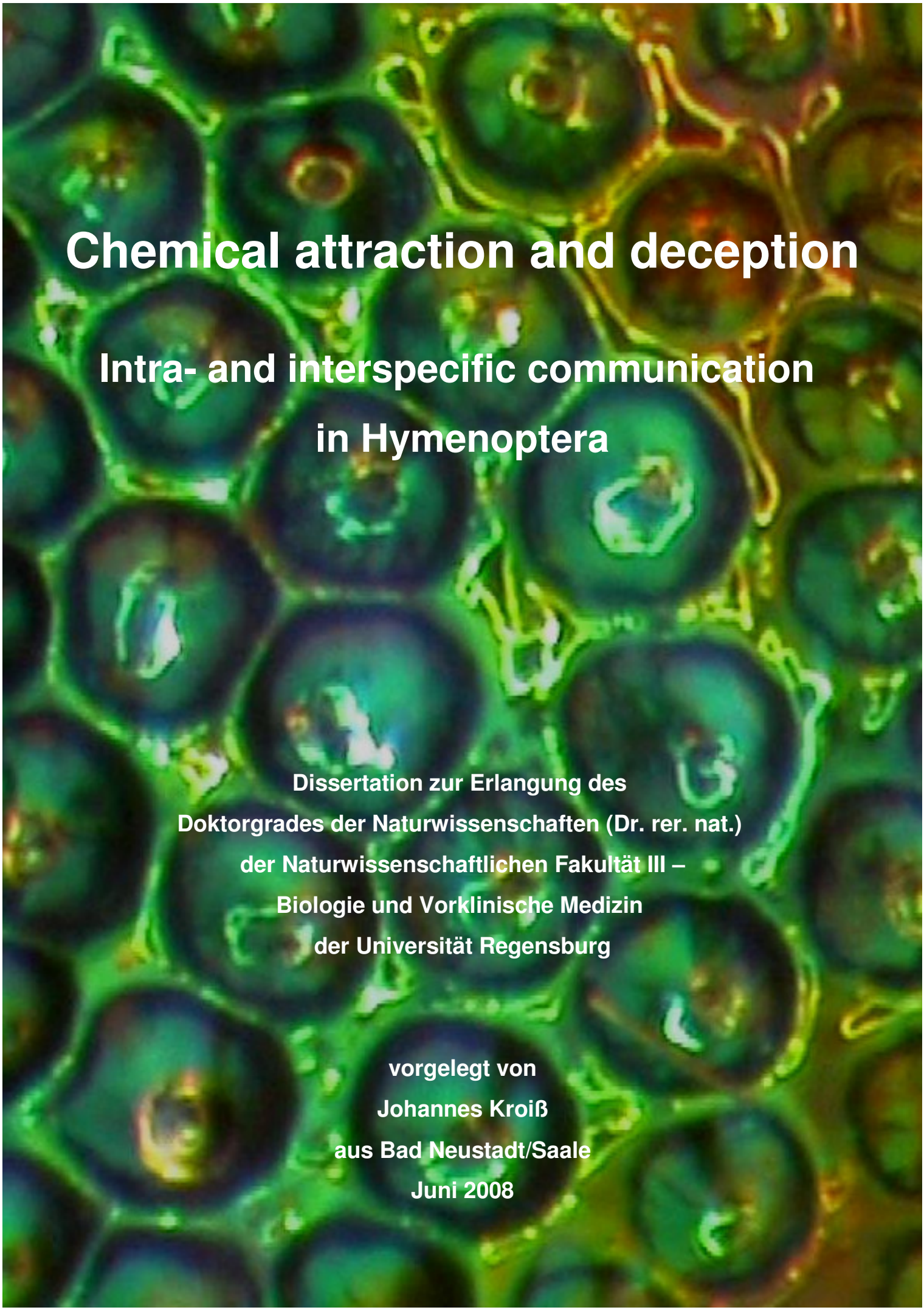

Chemical attraction and deception

Intra- and interspecific communication in Hymenoptera

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“I cannot persuade myself that a beneficent and omnipotent God would have designedly created the Ichneumonidae with the express intention of their feeding within the living bodies of Caterpillars.”

Charles Darwin, 22 May 1860, Letter to Asa Gray

CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following manuscripts:

Kroiss J, Schmitt T, Schreier P, Strohm E, Herzner G. 2006. A selfish function of a "social" gland? A postpharyngeal gland functions as sex pheromone reservoir in males of a solitary wasp. Journal of Chemical Ecology 32:2763-2776 (chapter 2).

Herzner G, Goettler W, Kroiss J, Purea A, Webb A, Jakob P, Roessler W, Strohm E. 2007. Males of a solitary wasp possess a postpharyngeal gland. Arthropod Structure & Development 36:123-133 (chapter 3).

Kaltenpoth M, Kroiss J, Strohm E. 2007. The odor of origin: Kinship and geographical distance are reflected in the marking pheromone of male beewolves (*Philanthus triangulum*, Hymenoptera, Crabronidae). BMC Ecology 7:11 (chapter 4).

Kroiss J, Strohm E. in preparation. Male territoriality and mating system in the European beewolf *Philanthus triangulum* F. (Hymenoptera, Crabronidae) – evidence for a “hotspot” lek polygyny. (chapter 5).

Kroiss J, Schmitt T, Göttler W, Schreier P, Strohm E. in preparation. Do males of the cuckoo wasp *Hedychrum rutilans* (Hymenoptera, Chrysididae) have a sex pheromone? (chapter 6).

Kroiss J, Bordon S, Strohm E. in press. Hydrocarbons in the nest material of a solitary digger wasp represent a kairomone for a specialized the cuckoo wasp. Animal Behaviour (chapter 7).

Strohm E, Kroiss J, Herzner G, Laurien-Kehnen C, Bohland W, Schreier P, Schmitt T. 2008. A cuckoo in wolves' clothing? Chemical mimicry in a specialized cuckoo wasp of the European beewolf (Hymenoptera, Chrysididae and Crabronidae). Frontiers in Zoology. 5:2 (chapter 8).

Kroiss J. 2008. Chemische Tarnkappe bei parasitoiden Goldwespen. Naturwissenschaftliche Rundschau. 61: 191-193 (chapter 9).

Kroiss J, Schmitt T, Strohm E. in press. Low level of cuticular hydrocarbons in a parasitoid of a solitary digger wasp and its potential for concealment. Entomological Science (chapter 10).

Kroiss J, Strohm E, Vigneron JP. submitted. A cuticular multilayer generates the iridescent coloration in chrysidid wasps (Hymenoptera, Chrysididae). Journal of the Royal Society Interface (chapter 11).

CHAPTER 1

GENERAL INTRODUCTION

Communication is a crucial process for both intra- and interspecific interactions of all organisms. Likewise it has gathered great interest in biological research, in particular in behavioral and evolutionary ecology. This thesis aims to contribute to the knowledge of intra- and interspecific chemical communication in the Hymenoptera. The first part of the thesis will deal with the evolution of the sex pheromone of males of a digger wasp, the European beewolves (*Philanthus triangulum*, Hymenoptera, Crabronidae) and its potential for female choice. The second part addresses the significance of cuticular hydrocarbons for the interactions of beewolf females with the specialized cuckoo wasp *Hedychrum rutilans* (Hymenoptera, Chrysididae). Chapter 1 will shortly summarize the current knowledge about intra- and interspecific chemical communication. The first part of the chapter will focus on sex pheromones and their potential for female choice in the context of sexual selection theory and will be complemented by information about the evolution of lek mating-systems. The second part will focus on the significance of cuticular hydrocarbons for interspecific chemical communication and will be supplemented with a short introduction to structural colors in nature. Chapter 1 will be closed with an overview of the biology of the two model organisms.

1.1 CHEMICAL COMMUNICATION

Both intra- and interspecific interactions require some kind of communication. Intraspecific communication may be essential for courtship, territorial, and cooperative or social behavior. Interspecific communication might involve warning of predators, attraction of pollinators, or host-finding of parasites or parasitoids. So far, several attempts have been made to define communication from different perspectives (Hauser 1996). Wilson (1975) defined biological communication as “the action on the part of one organism (or cell) that alters the probability pattern of behavior in another organism (or cell) in a fashion adaptive to either one or both of the participants. By adaptive I mean that the signalling, or the response, or both, have been genetically programmed to some extent by natural selection”. This rather broad definition is now widely used and accepted in biology. The mode of communication and the sensory channels used vary considerably in response to the environment and the sensory equipment

of the organisms. Communication might involve visual (Harder et al. 1996), acoustic (Catchpole & Slater 1995; Fitch 2000; Petitto 2000; Ryan 1985; Wagner & Reiser 2000), tactile and vibratory (Hill 2001), electric (Hopkins 1988), or chemical signals (Jackson & Morgan 1993; Johansson & Jones 2007; Johnston 2003).

Some definitions (Hauser 1996) and the everyday use of the word "communication" imply to some extend that both the sender and the receiver benefit from the signal and that the act of communication is adaptive for both participants. This might in fact apply to some intraspecific cases of communication like honest sexual advertisement or recruitment of nestmates in social insects. Correspondingly in interspecific communication, the aposematic coloration of certain organisms signalling unpalatability or toxicity is adaptive both for the sender and the receiver of the signal. However, there are many cases in which the sender tries to affect or deceive the receiver of the signal. This might be true for interspecific interactions, when prey species deceive predators with regard to their defensive abilities, predators deceptively attract prey, flowers lure pollinators, or parasites acquire resources or brood care from their hosts by imitating visual (Bates 1862; Brooke & Davies 1988; Greene et al. 1987; Lloyd 1965; Lloyd 1975; Lloyd 1986; Malcolm 1990), tactile (Hölldobler 1967; Hölldobler 1967; Hölldobler 1976), or chemical signals (Dettner & Liepert 1994; Gemeno et al. 2000; Haynes et al. 2002; Schiestl et al. 1999; Yeargan 1994; see Haynes & Yeargan 1999 for a review). Moreover, even many intraspecific interactions are not free of conflict. For example, there is a conflict between the sexes (Andersson & Simmons 2006; Chapman et al. 2003; Gavrilets et al. 2001; Parker 1979) and males might want to mislead females with regard to their own quality.

Hence it is evident that communication may or may not be advantageous for both the sender *and* the receiver of the signal. Therefore, communication is either cooperative or non-cooperative and signals are honest or deceitful.

1.2 SEMIOCHEMICALS

Communication by means of chemical substances has a great importance in many groups of organisms. Chemical communication is especially important for intra- and interspecific communication in insects. Both solitary and social insects release many different substances from diverse glands with various functions, making them "a walking battery of exocrine glands" (Hölldobler & Wilson 1990). However, chemical communication is hard to grasp for

us humans, because in our communication we predominantly rely on visual and acoustical cues and we do not possess highly developed olfactory organs, at least we are not aware of some of our olfactory perceptions. To circumvent the limitations of human chemical senses, several techniques have been established to study the chemical nature of the compounds and their physiological and behavioral effects (e.g. gas chromatography (GC), coupled gas chromatography – mass spectrometry (GC-MS), GC – electro-antennal-detection (GC-EAD), and bioassays). Organic compounds serving for intra- or interspecific communication have been defined as "semiochemicals" (Regnier 1971) and can be subclassified in pheromones, i.e. substances involved in intraspecific communication, and allelochemicals, i.e. substances utilized for interspecific communication.

1.3 INTRASPECIFIC CHEMICAL COMMUNICATION – PHEROMONES

1.3.1 Overview over pheromone communication in insects

Pheromones are chemical substances serving for intraspecific chemical communication (Karlson & Lüscher 1959). Chemical communication is thought to represent the most ancient form of communication (Bradburry & Vehrenkamp 1998) and pheromones have been described for nearly all kinds of organisms from bacteria (Ben Jacob et al. 2004) to fungi (Casselton 2002), plants (Sekimoto 2005), and higher animals (Bradburry & Vehrenkamp 1998; Wyatt 2003). Insects probably represent the group of animals with the most diverse array of pheromones. Pheromones have been shown to play a role e.g. for trail marking (Blatrix et al. 2002; Keeling et al. 2004; Steinmetz et al. 2002) and marking of territories (Wenseleers et al. 2002) and as alarm (Byers 2005; Fortunato et al. 2004; Free et al. 1983; Hölldobler 1995; Hölldobler & Wilson 1990; Mondor & Roitberg 2003), aggregation (Schaner et al. 1987; Torto et al. 1994), or sex pheromones (Ayasse 2001; Ferveur 2005; Johansson & Jones 2007; Keeling et al. 2004). In all these cases, the pheromone entails an immediate behavioral response ("releaser pheromone"). In contrast, there are also pheromones inducing persistent physiological changes like the "queen substance" of honeybees ("primer pheromone"; Wilson 1971).

Sex pheromones serve to attract conspecifics of the other sex for mating. Both females and males might produce such pheromones and the pheromones might adopt diverse functions in the process of mating (Johansson & Jones 2007). Female sex pheromones of insects have

attracted a lot of attention in the last four decades of pheromone research, since they have the impressive potential to attract males over several kilometers and are important in the field of pest management (Howse et al. 1998). However, due to the asymmetry between the sexes (Trivers 1972), male and female sex pheromones are expected to underlie largely different selective pressures (Phelan 1992; Phelan 1997) and male sex pheromones might not only play a role for long-distance mate attraction as well as species and mate recognition, but, more importantly, in the context of mate assessment (Johansson & Jones 2007).

1.3.2 Sexual selection and mate choice

When Charles Darwin proposed the theory of evolution by means of natural selection, he already noticed that "...natural selection has been the most important, but not the exclusive, means of modification" (Darwin 1859). Darwin was especially puzzled about the evolution of conspicuous male traits like bird song, elaborate ornaments, and male weaponry. These traits are expected to be selected against by natural selection and should therefore be eliminated by natural selection (Andersson 1994).

"Sexual selection [...] depends, not on a struggle for existence, but on a struggle between the males for possession of the females; the result is not death to the unsuccessful competitor, but few or no offspring. [...] Thus it is, as I believe, that when the males and females of any animal have the same general habits of life, but differ in structure, colour, or ornament, such differences have been mainly caused by sexual selection" (Darwin 1859).

Sexual selection – resulting in either male-male contest and appropriate weaponry or female choice according to male ornaments – is the result of the asymmetry in interest between the sexes (Trivers 1972). Females on the one hand produce few, large, and therefore costly eggs and invest a large amount of resources and time in raising the young. Males, on the other hand, produce a lot of small and therefore cheap sperm and mostly invest a very low amount of resources and time in breeding. Therefore, the reproductive success of females is limited by the amount of resources available for the production of the costly eggs and for the brood care. As a result, females should be choosy when it comes to mating and they are expected to choose the best male available to maximize their reproductive success. Male reproductive success, however, is limited by the number of mates. Consequentially, males can maximize their reproductive success by attracting and mating with as many females as possible.

Males can attract receptive females by advertising their quality using elaborate ornaments. These complex ornaments provide the female with information about species affiliation and additionally with information about the ability of a male to provide direct or indirect benefits for the offspring. However, the elaborate ornaments and the signals sent by the males to advertise their quality have to be "honest" because otherwise females may be fooled by cheaters. Therefore, the ornaments have to be costly to develop and maintain (i.e. a handicap). Only genuinely good quality males are able to express the signal (Zahavi 1975; Zahavi 1977). Dishonest signals will not be used by females for mate choice and will fall into disuse.

A female might benefit directly (i.e. non-genetically) from mating with a certain male when males differ in their ability to provide brood care or resources (Halliday 1983; Vahed 1998) or in their parasite or disease load (Clayton 1991; Hamilton & Zuk 1982). Direct benefits do not play a role in beewolves and will not be treated in detail. As an alternative to direct benefits, a female may benefit indirectly from mating with a high quality male, when the offspring quality depends on the genetic background of the mate. Several models have been suggested to explain female mate choice according to genetic benefits. The most prominent of these models is the "good genes" model (Andersson 1994; Hine et al. 2002; Johnstone 1995; Møller & Alatalo 1999; Wilkinson et al. 1998). This model predicts the existence of a single best male within a population, which is the best choice for all females. Qualitative or quantitative aspects (i.e. the intensity) of a sexual signal might play a role for mate choice according to a good genes model. The intensity of a sexual signal may be for example the volume of bird song or the intensity of courtship display. The alternative model of the best compatibility/complementarity on the other hand, predicts that a particular male is the best choice for a particular female (Colegrave et al. 2002; Halliday 1983; Johnsen et al. 2000; Mays & Hill 2004; Neff & Pitcher 2005; Reinhold 2002; Tregenza & Wedell 2000). Qualitative and quantitative aspects of a sexual signal, for example the composition of a complex pheromone, might indicate the genetic background of a potential mate.

Males with "good genes" might stand out against other males with regard to their resistance to parasites, their metabolic rate, or their ability to escape predators. Males can signal their physiological superiority, when they are able to maintain exaggerated and expensive ornaments (Doty & Welch 2001; Kokko 1998; Proulx et al. 2002; Thompson et al. 1997). Such a female choice according to a "good genes" model has been shown in many animal species (Møller & Alatalo 1999; Welch et al. 1998). Nevertheless, it has to be kept in mind that the "good genes" theory has some pitfalls, especially in the context of sexually antagonistic (SA) genes. These SA genes have opposite fitness effects when expressed in males as opposed to females and might weaken the potential indirect genetic benefits of sexual selection by

reducing the fitness of opposite-sex progeny from high-fitness parents (Arnqvist & Rowe 2002; Chippindale et al. 2001; Oneal et al. 2007; Pischedda & Chippindale 2006; Rice 1992). However, the degree to which SA genes are expected depends to a high degree on the mode of sex-determination (Albert & Otto 2005; Gibson et al. 2002).

The compatibility of a male depends amongst other on the degree of relatedness. The relatedness can range from strict inbreeding to strict outbreeding, both of which can have certain advantages and disadvantages. It has long been realized that outbreeding has profound disadvantages and that "the grossest blunder in sexual preference [...] would be to mate with a species different from its own and with which hybrids are either infertile or [...] at so serious a disadvantage as to leave no descendants" (Fisher 1930; see also Aspi 2000; Edmands 1999; Palmer & Edmands 2000; Partridge 1983; Peer & Taborskyi 2005; Sagvik et al. 2005). Similarly, inbreeding also has disadvantages for reproduction (inbreeding depression: Bischof 1972; Palmer & Edmands 2000; Pusey & Wolf 1996). The model of "optimal outbreeding" suggests that females should choose a mate of intermediate genetic distance to balance the costs of in- and outbreeding (Bateson 1978; Bateson 1980; Bateson 1983). While it has been shown that several animal species avoid both in- (Brown & Eklund 1994; Gerlach & Lysiak 2006; Pusey & Wolf 1996; but see Keller & Fournier 2002) and outbreeding (Harmsen & McKay 1985), information about mate choice according to a model of optimal outbreeding is lacking. However, mate choice for heterozygous or genetic dissimilar mates has been demonstrated (Hoffman et al. 2007).

In Hymenoptera, knowledge about outbreeding depression is relatively scarce. Inbreeding, however, can have an especially large disadvantage. While deleterious mutations that might be disadvantageous in diploid organisms are usually erased very quickly due to the haploidy of males (Goldstein 1994; Henter 2003), inbreeding has a great disadvantage in most Hymenoptera due to the prevailing mechanism of sex-determination, the single-locus complementary sex determination (sl-csd) (Beye et al. 2003; Cook 1993; Haig 1998). Usually, males develop from unfertilized eggs whereas females develop from fertilized eggs. However, if fertilized eggs are homozygous at the sex-determination locus, eggs will not develop into females, but into mostly infertile males (Cook & Crozier 1995; Cook 1993; Owen & Packer 1994; but see Cowen & Stahlhut 2004). Since the probability that this locus becomes homozygous is greatly increased by matings among relatives, the costs of inbreeding are especially high in Hymenoptera with sl-csd. Thus, females should be strongly selected to avoid mating with closely related males.

1.3.3 Sex pheromones and mate choice

Mate choice according to male ornamental traits and genetic benefits has been studied in great detail. However, most studies have focused on acoustical and visual characters like bird-song, frog calls, and bird plumage (Andersson 1994; Bennett et al. 1996; Møller & Alatalo 1999; Ryan 1983; Searcy 1992). Much less emphasis has been placed on mate choice according to chemical signals (Eisner & Meinwald 1995; Hine et al. 2002; Ings et al. 2005; Johansson et al. 2005; Moore 1997; Sappington & Taylor 1990; Sappington & Taylor 1990; Sappington & Taylor 1990; Vainikka et al. 2006; Van Dongen et al. 1998). Though mate choice by means of pheromones is probably very widespread in nature, this is not surprising, since the technical requirements for individual analysis of pheromone characteristics have only been available since the last few decades. However, due to the great quantitative and qualitative variability, chemical signals have a huge potential for carrying large amounts of information for mate choice (Angioy et al. 2003; Ayasse 2001; Hölldobler 1995; Lemaster & Mason 2002). Correspondingly, evidence is now accumulating, that male sex pheromones can play an important role for female mate choice (see Johansson & Jones 2007 for a review). The function of sex pheromones can be very diverse and pheromones might play a role for species recognition, mate recognition, and mate assessment. These levels of mate choice, however, constitute a continuum and are not mutually exclusive (Ryan & Rand 1993). While in the early years of pheromone research long-distance mate attraction, species-, and mate recognition has attracted most interest, research is now also extending to the significance of pheromones for mate assessment.

There are three prerequisites for the suitability of pheromones for mate assessment. First, the pheromone has to vary among individuals, either quantitatively or qualitatively, thus making individual mate choice possible (Eltz et al. 2005; Eltz et al. 2003; Eltz et al. 1999; Johansson & Jones 2007). Second, the production and the delivery of the pheromone have to inflict costs on the producer to ensure honesty of the signal (Zahavi 1975; Zahavi 1977). The expression of costly signals is usually condition-dependent and individuals of good quality are better able to afford exaggeration of the signal (Johansson et al. 2005; Rantala et al. 2003). Third, the additive genetic variance of “pheromone traits” should be relatively high as compared to traits more important to survival. Sex pheromones of several organisms have been shown to meet these three requirements (Johansson & Jones 2007).

Females have been shown to benefit from choosing a male with certain pheromone characters. In some species, sex pheromones may serve as either a direct resource or as an indicator of a resource (Johansson & Jones 2007). Pheromones serving as a direct resource

mostly consist of plant-derived substances that primarily serve to protect the insects from predators. These substances are usually not only emitted as a pheromone but also transferred via a spermatophore, and the offspring gains a direct benefit from obtaining a large amount of the substance (Gullan & Cranston 2004; Landolt & Phillips 1997). Alternatively, pheromones may provide information about the ability of a male to provide a resource, like shelter in the American lobster (Atema 1986; Atema 1995). Third, pheromones might also provide information about the fertility of a male (Johansson & Jones 2007).

In other species, however, sex pheromones might serve as an indicator of indirect, i.e. genetic benefits. In this case, a female might benefit from discriminating among males and choosing a good or suitable male through increased fitness of its offspring. Females might choose a male according to the "good genes" model, assuming that the male sex pheromone comprises an exaggerated, condition-dependent chemical signal. So far, evidence for mate choice by means of sex pheromones according to a "good genes" model is scarce (Rantala et al. 2003), as compared to mate choice by means of visual or acoustical characteristics. Alternatively, sex pheromones might serve as an indicator of compatibility and females might benefit from choosing a genetically dissimilar mate (Tregenza & Wedell 2000), thus balancing the costs of in- and outbreeding. Pheromones have been shown to provide good means to assess genotypes (Mays & Hill 2004; Penn 2002; see also Johnston 2003; Wedekind et al. 1995; Zelano & Edwards 2002). Research has so far focused on the MHC-genes and female preference for dissimilar MHC-genes has been demonstrated for several vertebrate taxa like mice (Penn & Potts 1998), humans (Thornhill et al. 2003; Wedekind & Furi 1997; Wedekind et al. 1995), newts (Garner & Schmidt 2003), sticklebacks (Milinski 2003; Reusch et al. 2001), and guppies (Shohet & Watt 2004). However, in these experiments the relative importance of learning and innate preference is not always clarified. Mate choice according to a model of "optimal outbreeding" requires interindividual variation of the pheromone with kinship and geographic distribution. While there are several reports on geographical variation of female sex pheromones (Gemeno et al. 2000; Huang et al. 1998; McElfresh & Millar 2001; Vereecken et al. 2007; Yatsynin et al. 1996), there are only few report on geographical variation of male sex pheromones (Aldrich et al. 1989; Hamilton et al. 2005; Ryan et al. 1995; Watts et al. 2005) and consequently, mate choice by means of a sex pheromone according to an "optimal outbreeding" model has not been shown so far. There are some pieces of evidence suggesting that multi-component sex pheromones might contain information about kinship and might therefore allow recognition of close relatives and as a consequence inbreeding avoidance (Herzner et al. 2006).

It has to be kept in mind that it is in principal possible that females choose their mate according to several aspects at the same time, for example according to a "good genes" model

and according to the genetic compatibility (Mays & Hill 2004; Neff & Pitcher 2005; van Doorn & Weissing 2004). It has been questioned whether mate choice according to several genetic aspects is possible. However, the conflict between the two aspects is expected to be not extraordinarily high (Mays & Hill 2004).

As a summary, evidence is accumulating that male sex pheromones have a great potential as "loud" and exaggerated chemical signals, comparable with a "chemical peacock tail", but might also have the potential to signal genetic compatibility/complementarity.

1.3.4 Mating systems and Leks

Sexual selection theory, as pointed out in paragraph 1.3.2, has the potential to explain conspicuous male traits such as male ornaments or weapons for intrasexual conflict. However, the mating system of a given species may evolve in response to both sexual and natural selection. Emlen and Oring (1977) proposed that the distribution of females in time and space and the potential for monopolizing females accounts for the prevailing mating system (see also Choe & Crespi 1997). The distribution of females in time and space, however, is influenced by ecological conditions (see also Wickman & Rutowski 1999). According to the diverse ecological conditions, different mating systems have evolved. In some mating systems, there is a high potential for monopolizing access to females either by monopolizing the females themselves or by monopolizing resources essential for females. In these cases, a resource defence polygyny or a female defence polygyny is likely to evolve.

In other species, however, the potential to control exclusive access to females is low. In these cases, the prevailing mating system is a male dominance polygyny. In a male dominance polygyny, males sort out their relative position of dominance among themselves in a mating arena, where most matings occur. Males exhibit a flashy visual, acoustic, or chemical display. Such a mating system is defined as a "lek" polygyny. Males cluster in such a "lek" mating arena, where they occupy small territories, which do not contain any resources essential to females. Females visit such leks for the sole purpose of mating (Balmford 1991; Bradbury 1981; Bradbury & Gibson 1983) and they obtain only gametes by mating in leks (Höglund & Alatalo 1995; Shelly & Whittier 1997). Males do not provide any non-genetic, i.e. direct benefits like brood care or other resources to the females, but females might gain direct benefits, when males vary in their fertilization ability (see Droney 2001; Droney 2003 for a discussion).

Lek mating systems are characterized by two basal characteristics. First, males compete among themselves e.g. for a good position in the lek, and, second, females are expected to choose among males in a lek or among males in different leks (Droney 1992). Both male-male competition and female choice are the driving forces of sexual selection and therefore, the evolution of lek mating systems is of special interest for the understanding of sexual selection.

Males may use visual, acoustical, or chemical signals both to establish a hierarchy in the lek and to attract females (Höglund & Alatalo, 1995; Wiley, 1974; Bradbury, 1981; Bradbury, 1977; Shelly & Whittier, 1997). While visual and acoustical signals have drawn much attention over the last few decades (e.g. Alcock et al. 1989; Friedl & Klump 2005; Höglund & Alatalo 1995), less attention has been paid to the peculiarity of species, where males produce pheromones to attract females. However, the investigation of such systems is very fascinating and has the potential to give new and important insights into the evolution of lek mating systems, the factors affecting fighting success in male-male contests as well as in the evolution of the properties of male sex pheromones.

In insects, lekking behavior occurs only in a few taxa. However, some groups have now been under intense study (e.g. (Field et al. 2002; Jones et al. 2000; Jones et al. 1998; Shelly 2001). In some groups, lekking is also associated with the production of a male-specific pheromone, which is thought to play a role for female attraction and for mate assessment (Beani et al. 2002; Droney & Hock 1998; Johansson et al. 2005; Widemo & Johansson 2006).

1.3.5 The evolution of lekking behavior and the "lek paradox"

Despite the intense study of lekking behavior in numerous taxa, the evolution of leks in not yet completely understood. Several hypotheses for the evolution of lekking have been suggested, for example a female mating bias for large clusters and choice among males (Hutchinson 2005; Shelly 2001), the reduction of the predation risk in large clusters (Isvaran & St Mary 2003), the "hotshot" hypothesis stating that males cluster around a especially attractive male, and the "hotspot" hypothesis assuming that males cluster at sites, where a large number of females is likely to pass by (Droney 1994; Westcott 1994). However, the causes for lek-evolution might differ considerably between species, and within a single species, there might be a "complex mosaic of interacting ecological pressures" leading to the evolution of lekking behavior (Field et al. 2002).

Apart from the ultimate causes of lek evolution, there is another problem of leks. It has been assumed and demonstrated that there is a strong mating skew in a given lek (i.e. few males siring most of the populations' offspring) and females choose males with the most elaborate visual, acoustical, or chemical ornaments. This strong directional selection should lead to the loss of genetic variation with regard to the exaggerated male character. The paradoxical situation that there is still a large amount of variation in the expression of these ornaments despite of the strong directional selection has become famous as the "lek paradox" (Kirkpatrick & Ryan 1991; Kotiaho et al. 2001; Taylor & Williams 1982; Tomkins et al. 2004). As a resolution of this problem, it has been suggested that the expression of the signals used for female attraction are highly condition-dependently, which would maintain genetic variation (Droney 1996; but see Hine et al. 2004; Lee 2005). Additionally, depending on the condition of a male, alternative mating tactics like satellite behavior as opposed to territorial behavior may be adopted (Dominey 1984; Droney & Hock 1998). Although the reproductive success of a male adopting an alternative mating strategy may be lower compared to "normal" males, this phenomenon might also maintain genetic variation in a population.

In conclusion, many attempts have been made to explain the evolution of lek mating systems. However, the ultimate causes for lek evolution might differ considerably between species and analysis of new examples of lek mating systems, especially with chemical signals released by the competing males, might provide new and important insights into male-male competition and especially female choice.

1.4 INTERSPECIFIC CHEMICAL COMMUNICATION

Chemicals do not only play a role for intraspecific communication, but are also important for interspecific communication. These chemicals, called allelochemicals, are very diverse both in chemical structure and function. Allelochemicals can be classified depending on who benefits from the signal. In principal, either the sender of the signal, the receiver, or both can benefit from the signal. When both the sender and the receiver benefit from the signal, a substance is defined as a synomone. Synomones are for example substance emitted by plants to attract parasitoids or predators of herbivores in "tritrophic interactions" (Mattiacci et al. 1994; Smid et al. 2002).

Allelochemicals benefiting only the receiver of the signal are called kairomones (Brown et al. 1970; Nordlund 1981; Nordlund & Lewis 1976). Kairomones can be classified according to

their function, for example as enemy-avoidance kairomones, sexual kairomones, aggregation kairomones, and foraging kairomones (Ruther et al. 2002). Foraging kairomones are used by the benefiting organism in the context of resource location (Ruther et al. 2002) and have been intensively studied for parasitoid host location (Godfray 1994). Foraging kairomones may play a role during different steps of the foraging process like host habitat-location, host-location, host-recognition, and host-acceptance (Godfray 1994; Ruther et al. 2002). Kairomones might either be cues from the microhabitat of the host or the host plant. Substances emitted by plants to attract parasitoids of their herbivores ("tritrophic interactions"; see "synomones" above) might for example serve as foraging kairomones for the parasitoids (Schnee et al. 2006). Alternatively, fungi (Greany et al. 1977) or yeasts (Dicke 1988) associated with the host might produce substances that serve as foraging kairomones. Similarly, the presence of certain substances in host frass (Jones et al. 1971; Lewis 1970; Lewis & Jones 1971; Ruther & Steidle 2000) or honeydew (Ayal 1987; Bouchard & Cloutier 1984; Bouchard & Cloutier 1985) might be exploited by parasitoids for host location. Parasitoids might also use direct cues from the host. These can be either substances purposely emitted from the host for intra- or interspecific communications like sex pheromones (Mizutani 2006), defensive secretions (Geiselhardt et al. 2006; Godfray 1994), or substances located on the host cuticle (Colazza et al. 2007; Jones et al. 1973; Jones et al. 1971; Lewis et al. 1976; Morehead & Feener 2000; Muratori et al. 2006; Vinson et al. 1975). As a consequence, applied entomology and integrated pest management take advantage of the fact that parasitoids use olfactory cues for host location (Agelopoulos et al. 1999; Lewis & Martin 1990).

Allelochemicals that are advantageous to the sender and disadvantageous to the receiver are referred to as allomones. Chemicals beneficial for the sender are for example repellents or defensive secretions (Blum 1981; Post et al. 1984). In the same manner, substances emitted by a predator to attract prey, as it is the case in the "aggressive chemical mimicry" of Bolas spiders, are considered as allomones. In this case, Bolas spiders chemically mimic the pheromone of certain moth species and attracted male moths are caught by the spider (Gemeno et al. 2000; Haynes et al. 2002; Haynes & Yeargan 1999; Yeargan 1994). Likewise, some orchid species attract male bees or wasps by specifically imitating the particular female sex pheromones and exploit attracted males for pollination (Schiestl et al. 2000; Schiestl et al. 1999; Schiestl & Peakall 2005). One important and widespread example of allomones is "chemical mimicry". In this case, social parasites, parasitoids, or predators imitate the composition of the cuticular hydrocarbons (CHCs) of their host or prey and therefore, they are not recognized and attacked by their hosts. This phenomenon of chemical mimicry, which benefits the sender of the signal, will be treated in more detail in section 1.4.1.4. It is important to point out that in this case it is the deceptive intention of the sender of the signal

to be as inconspicuous as possbile as compared to the case of the aggressive chemical mimicry or the floral sex pheromone mimicry where the sender of the signal tries to attract the attention of the signal receiver.

1.4.1 Cuticular hydrocarbons (CHCs)

1.4.1.1 Chemical nature, biosynthesis, and primary function of CHCs

Cuticular hydrocarbons (CHCs) are substances that are located on the surface of the insect cuticle (Howard & Blomquist 1982; Howard & Blomquist 2005). Usually, insects possess a more or less complex mixture of CHCs. In most cases, the CHCs consist of alkanes, methyl-branched alkanes, alkenes, and – to a lesser degree – alkadienes and alkines. According to current knowledge, the hydrocarbons are synthesized in the oenocytes, loaded onto lipophorin, and transported to the fat body or the cuticle (Howard & Blomquist 2005; Young et al. 1999). In ants, a special gland, the postpharyngeal gland (PPG) might be involved in the storage and circulation of the CHCs among nestmates (Bagneres & Morgan 1991; Cabrera et al. 2004; Do Nascimento et al. 1993; Lucas et al. 2004; Soroker et al. 1995). The primary function of CHCs is to reduce the water loss of the insect body (Gibbs 1995; Hadley & Louw 1980; but see Gibbs et al. 2003). The substances represent a wax-like hydrophobic coating of the cuticle, which help to avoid desiccation, which is especially important in small organisms in arid habitats. CHCs might not only be found on the cuticle of insects, but also in the nest-material, especially in social insect species (Grasso et al. 2005; Layton & Espelie 1995).

1.4.1.2 CHC variability

Usually, the CHC composition is species-specific, which in some cases even allows scientists to discriminate different species according to their CHC profile (i.e. chemotaxonomy; Page et al. 1997). Besides that, the CHC composition might not only reflect species affiliation, but also environmental conditions (Nielsen et al. 1999; Noorman & Den Otter 2002), sex or age (Gibbs et al. 1995; Howard 1998; Panek et al. 2001), mating status (Johnson & Gibbs 2004), geographic origin (Dapporto et al. 2004; Uva et al. 2004), and potentially the infection with parasites (Dapporto 2007; Salvy et al. 2001).

1.4.1.3 Secondary function of CHCs for intra- and interspecific communication

Apart from the primary function for the reduction of water loss, CHCs have acquired several secondary functions. Most prominently, they play an important role for intra- and interspecific communication. In the context of intraspecific communication, they are crucial for nestmate recognition in social insects (Lorenzi et al. 2004; Ruther et al. 2002). Additionally, they sometimes function as sex pheromones (e.g. sex pheromones in *Drosophila*: Ferveur 2005; and *Colletes* bees: Mant et al. 2005). Furthermore in ants, they might provide information about the fertilityy or reproductive status of queens or workers, respectively (Dietemann et al. 2003; Endler et al. 2004; Liebig et al. 2000).

1.4.1.4 Chemical mimicry

Since the CHC composition usually varies between species, CHCs have the potential to play an important role also for interspecific communication. It has been shown that CHCs are important in the context of species recognition (Lucas et al. 2005; Takahashi & Gassa 1995) and predators or parasites are able to recognize and identify their prey or host species by means of their CHCs (Colazza et al. 2007; Howard et al. 1998). Accordingly, predators or parasites and parasitoids should be selected to evade chemical detection by their prey or host species. This is possible by qualitatively imitating the CHC composition of the respective prey or host species. This phenomenon is referred to as “chemical mimicry” (sensu lato; Dettner & Liepert 1994). When the CHCs are acquired by the mimic (i.e. the imitator of the CHCs) by direct contact with the host or the host nest, this is designated as “chemical camouflage” (Dettner & Liepert 1994). Only if the substances are produced by the mimic itself, one can talk about “real” “chemical mimicry (sensu stricto; Dettner & Liepert 1994).

Chemical mimicry has been shown for numerous species, especially in (social) parasites of social insects. Mostly, it is not completely clear, whether it is chemical mimicry or chemical camouflage. However, examples have been reported from many myrmecophilous species (Akino 2002; Akino et al. 1999; Elgar & Allan 2006; Elmes et al. 2002; Schlick-Steiner et al. 2004; Vander-Meer & Wojcik 1982), parasitic ant species (Lenoir et al. 2001; Lenoir et al. 1997), ant predators (Allan et al. 2002; Howard et al. 1990), parastoids (Akino & R 1998; Liepert & Dettner 1993; Liepert & Dettner 1996; Vandermeer et al. 1989), social parasites of paper wasps (Sledge et al. 2001; Turillazzi et al. 2000), *Varroa* mites (Martin et al. 2001), aphid predators (Lohman et al. 2006), and parasitic beetles (Geiselhardt et al. 2006). Thus,

chemical mimicry or camouflage has independently evolved in numerous insect taxa. However, it has not been shown in all cases, whether the CHCs are synthesized by the mimic or passively acquired from other individuals or nest-material. It is important to state that the chemical mimicry mentioned so far is in most cases equivalent to host mimicry like in the egg coloration mimicry of cuckoos (see Davies et al. 1989). Furthermore, most cases of chemical mimicry deal with species with a very narrow host or prey spectrum. Chemical mimicry systems might get highly complex when two or more host species are involved and might lead to a mosaic of chemical coevolution (Brandt et al. 2005; Nash et al. 2008).

1.4.1.5 Chemical insignificance

Apart from chemical mimicry, which is the qualitative imitation of the host CHCs, a second, quantitative approach to escape olfactory detectation has been described. In several studies, a very low amount of CHCs has been reported in social parasites or thievery ants and this CHC deficiency has been interpreted as a dilution of recognition cues (D'Ettorre & Errard 1998; Jeral et al. 1997; Lambardi et al. 2007; Lorenzi & Bagneres 2002). It has been suggested that this low amount of CHCs might represent a quantitative chemical strategy in a sense of a "chemical insignificance" (Lenoir et al. 2001). There is a growing body of evidence that a low amount of CHCs reduces the ability of insects to recognize conspecifics or nestmates (Lorenzi et al. 1999; Lorenzi et al. 2004; Panek et al. 2001). Accordingly, a low amount of CHCs of a parasite or parasitoid might reduce the probability of being detected by the host. Yet, it is not clear whether this CHC deficiency imposes an increased risk of water loss over the cuticle. Therefore, it is very interesting to take a closer look at the cuticle of species with a reduced amount of CHCs and to look for alternative adaptations of these species to avoid desiccation.

1.5 STRUCTURAL COLORS IN NATURE

1.5.1 Origin, proximate causes, and characterization of structural colors

Life on earth is colorful. Both animals and plants show an enormous variability of colors to attract mates or pollinators, deter enemies, or to hide from predators. The whole spectrum of

light from the ultraviolet to the infrared is used and some of the phenomena like polarized light (Sweeney et al. 2003) are not visible for the human eye. Colors in general have either a pigmentary origin or originate from the interaction of light with regular structures with the size of these structures being in the order of magnitude of the wavelength of visible light. While colors derived from pigments are more widespread in nature, so called structural colors are usually more attractive due to their intensity, the iridescence, and their color variation depending on the angle of vision.

Though the diversity of structures provoking colors is very high, it can be stated generally that structural colors originate from the interaction of periodic nanostructures with photons. These structures are able to affect the movement of photons. Depending on the lattice constant (i.e. the distance between the periods of the nanostructure) and the refractive indices (RI) of the two materials involved (see below) light of a certain wavelength is "not allowed" to propagate in the periodic nanostructure and will be totally reflected. Such a periodic nanostructure has been proposed to be a "photonic crystal" (John 1987; Yablonovitch 1987) and the periodicity of the structures can occur in one to three dimensions. The simplest case of such a photonic crystal is a multilayer (i.e. a 1D photonic crystal). Light of a certain wavelength will be reflected in one direction, the right angle to the surface of the multilayer. The reflected color will change from longer to shorter wavelengths as the angle of vision increases. A 2D photonic crystal has a periodicity in two dimensions and light will be reflected in two dimensions. The most complicated photonic crystal shows a periodicity in three dimensions and will reflect light of a certain wavelength in three dimensions (3D photonic crystal). Such a structure might be built for example by three-dimensional stacking of beams or spheres (Parker et al. 2003).

It is important to mention that within the periodic structure, the alternating layers have to have different RIs. Noticeable, the RI of biological substances is rather low as compared to materials like glass or metal. In insects, the prevailing material involved in the occurrence of structural colors is chitin. Chitin is the main substance of the cuticle of insects and all structural colors of insects originate in the cuticle or in derivatives of the cuticle. The average refractive index of chitin is 1.52 (Welch & Vigneron 2007). The RI of chitin can be higher if other substances like uric acid are embedded into the chitinous matrix (RI = 1.68, Vigneron, pers. comm.). Noticeable, the material with the highest RI involved in biological color-producing structures, is guanine with RI = 1.83 (Welch & Vigneron 2007; for comparison: glas RI = 1.45 – 2.14, titanium dioxide, an important white pigment RI = 2.71). In most cases, the second material of the periodic nanostructure is air. Air has a RI of 1.00 and therefore, the RI contrast in biological photonic structures is rather low (Welch & Vigneron 2007).

Photonic structures in biological materials can be investigated with different methods. First of all, reflectance spectra can be obtained to measure the wavelength of maximum reflectance. These reflectance spectra can be measured in the visible spectrum of light, the UV and the IR spectrum, and with different angles of incidence (Vigneron et al. 2006). After obtaining reflectance spectra of the structures of interest, one can predict the lattice constant of the periodic nanostructure responsible for the color origin. Afterwards, Scanning Electron Micrographs (SEM) should be taken from the structure. In the case of insect cuticle, the cuticle might be broken by freeze-fracture technique, thereby exposing the structure. Using SEM, the periodic nanostructures can be identified, and its properties (e.g. the dimensionality, the lattice constant, and the number of layers) can be measured and characterized. To complement the reflectance spectral analysis and the SEM characterization, one can model the optical properties (i.e. the reflectance spectrum) of the observed material taking the lattice constant, the proposed RIs, and the number of periodicities into account. If the results of the reflectance spectra, the characteristics obtained from the SEM, and the modeled reflectance spectra are consistent, one can be sure that the observed structure is responsible for the coloration (see Vigneron et al. 2006 for details).

1.5.2 Occurrence, function, and ultimate causes of structural colors

Most natural structural colors occur in animals (Vukusic & Sambles 2003) whereas structural colors can only very rarely be seen in plants (Lee 1991; Lee 1997; Lee & Lowry 1975; Vigneron et al. 2007) and in non-living matter like opal (Parker et al. 2003). Most reports on structural colors in nature deal with insects and among insects, butterflies are the classic example. Butterfly wings have drawn a lot of attention, especially during the last few years (Prum et al. 2006; Vukusic 2006; Vukusic et al. 1999; Wickham et al. 2006; Yoshioka & Kinoshita 2006; Yoshioka & Kinoshita 2006), but lately also other insect taxa like beetles (Parker et al. 1998; Parker et al. 2003; Vigneron et al. 2005; Vigneron et al. 2005; Vigneron et al. 2007; Welch et al. 2007) and damselflies (Vukusic et al. 2004) have been the subject of intensive studies. Structural colors can also be found in marine invertebrates (McPhedran et al. 2001; Parker et al. 2001; Welch et al. 2006) and vertebrates like fish (Bagnara et al. 2007), amphibians (Bagnara et al. 2007; Schmuek & Linsenmair 1988), birds (Doucet et al. 2006; Dresp & Langley 2006; Vigneron et al. 2006; Zi et al. 2003), and mammals (Prum & Torres 2004). Photonic structures can even be found in the fossil record with an age of up to 515 million years (Parker 2000; Parker 2004; Parker 2005; Parker & McKenzie 2003).

Though the before mentioned structural colors are very striking to the observer, the function of the coloration is not always clear. However, in classical examples of structural colors, males exhibit the colorful structures whereas females usually have a dull coloration and it is very likely that these structures have evolved for intraspecific signaling and more specifically for mate attraction. Therefore, these structures are probably a sexually selected trait and the result of female choice (Vukusic & Sambles 2003; Welch et al. 2007; Welch & Vigneron 2007; Zi et al. 2003). Recently, evidence has accumulated that these structural colors have also the potential to signal male quality (Kemp & Rutowski 2007; Kemp et al. 2006; Loyau et al. 2007).

In other cases, the function of the structural colors is not so clear. Sometimes, interspecific signaling (e.g. warning coloration/aposematism, mimicry, startling, crypsis) can be assumed as the ultimate cause for the coloration. Still, in other cases neither intra- nor interspecific communication seems to be the reason for coloration. The coloration might serve for thermoregulation (Biro et al. 2003; Kobelt & Linsenmair 1992; Koon & Crawford 2000; Schmuek & Linsenmair 1988) or the structures giving rise to the coloration have special mechanical properties and the colors are just a side-effect of the mechanical properties. However, if the optical appearance would be detrimental for the animal, it would probably be counterselected and the structures could either be covered with a pigment or the periodic structures could be tuned in a way that the reflection maximum turns into the ultraviolet or infrared spectrum. Recently, it has been recognized that the evolution of a color producing structure might be driven by several factors simultaneously, which would result in a multifunctional structure (Welch & Vigneron 2007). Due to the optical properties, natural photonic structures are subject to imitation by man, but so far, the success of this biomimetism is rather limited (Biro 2007; Chen 2001; Deparis 2006; Large et al. 2007; Vigneron et al. 2005).

1.6 BIOLOGY OF THE EUROPEAN BEEWOLF *PHILANTHUS TRIANGULUM* (HYMENOPTERA, CRABRONIDAE)

1.6.1 Systematic position of the European beewolf *Philanthus triangulum*

All species of digger wasps (Hymenoptera, Sphecidae and Crabronidae) hunt other arthropods as food for their larvae and nests are either built in the soil or in natural or self

constructed cavities like in wood or twigs. Digger wasps exhibit an amazing range of life history strategies and inhabit all continents except for Antarctica (Bohart & Menke 1976).

The genus *Philanthus* (Crabronidae, Philanthinae) comprises about 135 species (Bohart & Menke 1976). Most species occur in the palaearctis, palaeotropis, and the nearctis, but no *Philanthus* species inhabit the South American continent and Australia (Bohart & Menke 1976). *P. triangulum* has a very broad range of distribution. The European beewolf can be found in central and south Europe (Blösch 2000) including the British islands (Else 1995; Harvey & RG 1992; Jones 2000). The northern limit of its distribution is Sweden and Norway (Erlandsson 1962; Gunnardo 1992; Hansen 1997) and the distribution extends to the south over the whole African continent to South Africa including Madagascar (Simon-Thomas & Simon-Thomas 1980), and the Middle East (Bohart & Menke 1976).

1.6.2 Behavior of female European beewolves

European beewolf females exclusively hunt workers of the honeybee *Apis mellifera* as food for their larva. The hunting behavior of European beewolves is mediated by both visual (Tinbergen 1935) and olfactory cues (Herzner et al. 2003; Herzner et al. 2005). After beewolf females have discovered a honeybee feeding on a flower, they can be observed hovering in a distance of about 10 cm away from their prey before actually attacking the bee. This stage seems to be an important step in the hunting sequence of beewolves (Herzner et al. 2005) and honeybees appear to be olfactory identified during this phase of the hunting process. The substance that is crucial for the olfactory identification of the honeybees is the long-chained alcohol (*Z*)-11-eisosen-1-ol (Herzner et al. 2005). This substance has been shown to be present not only on the cuticle of honeybees but also in the surrounding air of foraging honeybees (Schmitt et al. 2007). Interestingly, this substance also constitutes the main substance of the sex pheromone of male beewolves (Schmidt et al. 1990; Schmitt et al. 2003). Potentially, male beewolves exploit the preexisting sensitivity of females for that substance, which has probably evolved in the context of hunting, for female attraction according to the sensory exploitation model (Herzner 2004; Herzner et al. 2005). Immediately after the attack, honeybees are paralyzed by injection of venom through the sting into the thorax of the honeybee (Dunbar & Piek 1982; Piek 1966; Rathmayer 1962; Rathmayer 1962).

Females build their nests in sandy soil, usually in areas with favorable microclimatic conditions. Beewolves will either nest in flat ground or – if available – in steep, sun-exposed slopes. Nowadays, beewolves are often colonizers of new habitats originating under

anthropogenic influence (see Evans 1974). The beewolf nest consists of a main burrow with up to 34 horizontal side burrows with one terminal brood cell each (Evans & O'Neill 1988; Simon-Thomas & Simon-Thomas 1972; Simon-Thomas & Veenendaal 1978; Strohm 1995). Beewolves use visual landmarks to find their own nest when returning from hunting or foraging (Beusekom 1948; Tinbergen 1932; Tinbergen 1938; Tinbergen & Kruyt 1938).

Fig. 1.1: Beewolf female carrying a honeybee at its nest entrance.

Beewolf females hunt up to six honeybees in succession (Fig. 1.1) and the bees are temporarily stored in the main burrow of the nest. Only after the bees are brought into the nest, a side burrow and a terminal brood cell is excavated and the bees are transferred into the brood cell. Each brood cell is provisioned with one to six honeybees. The provisioning behavior has been extensively studied and the amount of bees provided for individual offspring not only depends on the sex of the offspring, but e.g. also on the prevailing temperature, the availibility of honeybees, and the individual "performance" of the female (Strohm 1995; Strohm 1999; Strohm & Daniels 2003; Strohm & Linsenmair 1997; Strohm & Linsenmair 1997; Strohm & Linsenmair 1998; Strohm & Linsenmair 1999; Strohm & Linsenmair 2000; Strohm & Marliani 2002). After all prey items are transferred into the

brood cell, the beewolf female will lay an egg on top of one of the honeybees and will finally close the brood cell. Brood cells and the side burrow of the nest are always carefully closed after completion of the brood cell, most probably as a protection against parasitoids like cuckoo wasps and sarcophagid flies (Evans & O´Neill 1988). After hatching, the beewolf larva feeds on the prey items and after completion of the larval development, the larva will spin a bottle-shaped cocoon, which is attached to the distal side of the brood cell wall (Strohm 1995; Strohm & Linsenmair 1995). The larva will either overwinter and hatch in the following year or emerge later in the same year.

Since beewolf females build underground nests and the brood cells are usually very moist and warm, both the honeybee prey and the developing larvae are heavily threatened by fungal infestation (Strohm 2000; Strohm & Linsenmair 2001). Beewolf females have evolved several mechanisms to protect the brood cell content from this hazard. First, beewolf females cultivate symbiotic bacteria that are applied into the brood cell to reduce fungal infestation of the cocoon and therefore larval and pupal mortality (Kaltenpoth 2005; Kaltenpoth 2006; Kaltenpoth et al. 2006; Kaltenpoth et al. 2005). These bacteria of the genus *Streptomyces* are cultivated in antennal glands (Goettler et al. 2007; Kaltenpoth et al. 2005) and probably produce antimicrobial substances. The symbiotic bacteria are applied to the distal ceiling of the brood cell and additionally provide the developing larva with information about the position of the main burrow of the nest (Strohm & Linsenmair 1995). This allows the emerging imago to dig to the open main burrow and leave the natal nest without elevated costs for digging out. Apart from the symbiotic bacteria, beewolf females are able to protect the larval provisions by applying a secretion from the postpharyngeal gland (PPG) onto the honeybee prey. They thereby significantly slow down the onset of molding, most probably by reducing the incidence of water condensation on the honeybees (Herzner et al. 2007; Herzner & Strohm 2007; Herzner & Strohm accepted; Strohm & Linsenmair 2001).

1.6.3 Behavior of male European beewolves

Males of the European beewolf are well-known to show two peculiarioties. First, beewolf males establish small territories in the vicinity of female nests (Simon Thomas & Poorter 1972; Strohm 1995; Fig. 1.2). These territories do no contain any resources like flowers and are defended against other intruding males by aerial combats, mostly with no or few physical contact. However, males can sometimes be observed attacking and biting intruding males vigorously (K. Roeser-Mueller, pers. comm.). Territorial behavior is not very widespread in

insects, but nearly all *Philanthus* species exhibit territorial behavior (Alcock 1975; Evans & O´Neill 1988; Gwynne 1978; O´Neill 1979; O´Neill 1983). In many *Philanthus* species, the male territorial behavior and morphological features associated with territory ownership and fighting success have been studied. Mostly, male size is the most important factor for fighting success (O'Neill 1983; O'Neill 1983; O'Neill & Evans 1983). However, in *P. triangulum* males, size is not the crucial factor and no final conclusions about the feature determining territory ownership or fighting success have been drawn (Fregin et al. 2002; Strohm & Lechner 2000). Male territories are clustered in the vicinity of female nesting sites. These clusters of male territories constitute lek systems, where females have the opportunity to choose among the territorial males according to territory ownership or the position within the lek.

Fig. 1.2: Beewolf male perching in its territory.

Besides the territorial behavior, all *Philanthus* species (except for *P. albopilosus*) are characterized by the presence of a male sex pheromone. This pheromone was thought to be produced in a mandibular gland (Evans & O'Neill 1978; Gwynne 1980; McDaniel et al. 1987; McDaniel et al. 1992; O'Neill & Evans 1983; Schmidt et al. 1990; Schmidt et al. 1985) and is applied onto the territory substrate with a clypeal brush (Evans & O´Neill 1988). There is convincing evidence that receptive females are attracted into the male territories by the pheromone (Evans & O'Neill 1991; Evans & O´Neill 1988). Mating takes place or at least starts within a territory (Simon Thomas & Poorter 1972; Strohm 1995) and copulations seem to be under the control of beewolf females, since they are able to refuse mating due to their

larger body size (Evans & O'Neill 1988) or by bending their abdomen tip downwards therefore preventing a male from mating (E. Strohm, personal observation). Since no visual display of beewolf males can be observed and copulations usually start immediately after a female enters a territory, any form of female choice must be ate least predominantly based on pheromone characteristics.

The sex pheromone of *P. triangulum* has been characterized and comprises a complex blend of several substances with (*Z*)-11-eicosen-1-ol as the main compound (Schmidt et al. 1985; Schmitt et al. 2003). It has been shown in the last few years that the beewolf pheromone shows considerable interindividual variation. It has been demonstrated that the composition of the pheromone varies with the age of the males (Kaltenpoth & Strohm 2006). Furthermore it has been shown that the pheromone of beewolf brothers is more similar as compared to unrelated individuals (Herzner et al. 2006). Though it has not been demonstrated yet, this interindividual variation might allow the beewolf females to choose a mate according to a "good genes" model or according to a model of "good compatibility".

1.6.4 Mate choice in European beewolves

Female mate choice is expected to be especially important in beewolves. First, beewolf females mate only once (Evans & O´Neill 1988) and mating with a male of low genetic quality will affect all daughters of the female. Since beewolves are haplo-diploid, only daughters will inherit the genes of the females' mate, because males develop from unfertilized eggs. Females can probably gain a great advantage from choosing a good mate. Criteria for mate choice could be either territory ownership and therefore flying and fighting abilities, quantitative and qualitative criteria of the sex pheromone, or both. The performance and fitness of a beewolf female heavily depends on the ability to carry honeybees in flight into the nest (Strohm & Daniels 2003). A beewolf female could therefore benefit from choosing a male that has successfully established and defended a territory and therefore proven to have good flying abilities.

Additionally, females could choose a mate according to quantitative criteria of the beewolf pheromone. One of the results of this thesis is that the beewolf pheromone is stored in the same gland that serves for the storage of the secretion for the honeybee treatment against molding, the postpharyngeal gland (see chapters 2, 3, and 12). Protection of the offspring and its provision from molding and therefore the fitness of a female heavily depends on their ability to apply large amounts of this secretion onto the honeybees (Herzner & Strohm 2007).

Therefore, a beewolf female could probably benefit from mating with a male that produces large amounts of the pheromone, thus proving good biosynthetic abilities and a good energetic condition.

Beewolf females might additionally choose according to qualitative aspects of the male sex pheromone. Inbreeding is expected to impose extraordinarily high fitness costs onto the females (Herzner 2004; Herzner et al. 2006) and females should be selected to avoid matings with closely related males. Additionally, females might be selected to avoid matings with very distantly related males, for example to avoid the break-up of local adaptations against parasitoids. This might lead to the choice of a mate of intermediate genetic distance according to the optimal outbreeding model.

So far, the mating system of the European beewolf has not been characterized in detail. Since male territories cluster in the vicinity of female nesting sites and male territories do not contain resources essential for females like flowers one might assume a lek polygyny. However, no systematic study has been conducted to elucidate the mating system of European beewolves. In some North American species, the mating system has been studied, but new statistical methods might reveal different results and allow new interpretations (Evans & O'Neill 1988; Gwynne 1980).

1.6.5 Enemies and parasites of beewolves

The European beewolf and especially the brood cell containing the larva and its provisions is the target of several parasitoids. The most important parasitoid is the cuckoo wasp *Hedychrum rutilans* (Hymenoptera, Chrysididae). The biology of this parasitoid will be described in detail in the paragraph 1.7. Apart from *H. rutilans*, several other cuckoo wasps have been suggested to be parasitoids of *P. triangulum*. However, reports on *H. gerstaeckeri* (Kunz 1994; Olberg 1953), *H. nobile* (Aerts 1955), *Omalus auratus* (Morgan 1984; Olberg 1953), and *Chrysis ignita* (Olberg 1953) as beewolf parasitoids are not well supported.

Apart from cuckoo wasps, sarcophagid flies are important parasitoids of beewolves (Blösch 2000). Several species have been reported e.g. *Metopia agryocephala* (syn: *M. leucocephala*) (Olberg 1953; Strohm & Linsenmair 2000), *Miltogramma punctatum* (Strohm & Linsenmair 2000), *Phrosinella spp.* (Evans & O'Neill 1988), *Hilarella stitica* (Strohm & Linsenmair 2000), *Senatainia tricuspis* (Strohm & Linsenmair 2000), *Specapate albifrons* (Olberg

1953), and *Specapate conica* (Olberg 1953). Most likely, these species have a relatively broad host spectrum and are not specialized on the European beewolf (Larsson 1986; Lindner 1949; Olberg 1953). Like other insects, adult beewolves have many other unspecific predators like birds, lizards, asilid flies, and spiders (Evans & O'Neill 1988; Gunnardo 1992; Olberg 1953; Fig. 1.3). Additionally, adult beewolves may be attacked by parasitic conopid flies (*Psycocephala chrysorrhoea* and *P. vittata*), which lay their eggs into the abdomen of adult beewolves and conopid larvae will develop inside the abdomen (Olberg 1953; Simon Thomas & Poorter 1972).

Fig. 1.3: Beewolf male caught by a crab spider.

1.7 BIOLOGY OF THE CUCKOO WASP *HEDYCHRUM RUTILANS* (HYMENOPTERA, CHRYSIDIDAE)

1.7.1 Systematic position of the cuckoo wasp *Hedychrum rutilans*

Chrysidid wasps are a hymenopteran family comprising about 2440 described species (Kimsey & Bohart 1990). All species are parasitoids or cleptoparasites. To account for this parasitic lifestyle, this insect family is commonly known as "cuckoo wasps". Hosts of chrysidid wasps are usually other aculeate hymenoptera (Sphecidae, Eumenidae, Masaridae, Anthophoridae, Megachilidae, Halictidae, Apidae, Limacodidae) (Kimsey & Bohart 1990). Merely some basal families of the Chrysididae (Amesiginae, Loboscelidiinae) and very few species of the Chrysidinae are parasitoids of Tenthredinidae, Phasmatidae, and butterflies (Kimsey & Bohart 1990; Parker 1936). Chrysidid wasps exhibit a worldwide distribution except for Antarctica.

The genus *Hedychrum* covers about 146 species and belongs to the subfamily Chrysidinae (tribus Elampini; Kimsey & Bohart 1990; Niehuis & Wägele 2004). This genus is widely distributed over the whole Palaearctis, Africa, Madagascar, the Oriental region, and the Americas. Hosts of *Hedychrum* species are rarely recorded and are confined to the digger wasps subfamily Philanthinae (Hymenoptera, Crabronidae, Philantinae; Kimsey & Bohart 1990).

1.7.2 Coloration

Most cuckoo wasps exhibit a very spectacular and attractive appearance: generally, the whole body shows complete iridescent and metallic colors. Correspondingly, chrysidid wasps are also referred to as "golden wasps". The colors cover the whole visible spectrum of light. In the basal subfamilies Loboscelidinae and Allocoelinae however, metallic coloration is completely absent and the wasp coloration ranges from black to brown or reddish. Amiseginae are only partially metallic. The coloration of the genus *Cleptes* (Cleptinae) is very variable and ranges from non-metallic black to complete iridescence. The genera *Hedychridium* (Elampini) and *Parnopes* (Parnopini) are known to have species with non-metallic body parts, mostly the abdomen (Kimsey & Bohart 1990).

Interestingly, the prevalence of certain colors does not only vary with phylogenetic relationship but also with biogeographic patterns: for instance, cuckoo wasps in the Americas and Africa tend to be blue or green, palaearctic species in contrast are usually bicolored with a blue or green thorax and a brassy to coppery abdomen (Kimsey & Bohart 1990).

1.7.3 *Hedychrum rutilans*

The cuckoo wasp *Hedychrum rutilans* can be found in Europe, the oriental region, and northern Africa. This species belongs to the subfamily Elampini (Kimsey & Bohart 1990) and is sometimes also referred to as *H. intermedium*. Up to 10 subspecies have been described so far and therefore, the taxonomy of this species is sometimes rather confusing (Linsenmaier 1959; Linsenmaier 1968; Linsenmaier 1997).

Males and females are 6 - 10 mm long and size is not a good distinctive feature of the sexes. The appealing appearance of *H. rutilans* is impressively reflected in its scientific name standing for a "glossy-red perfume". The abdomen is single-colored red and thorax and head are bluish green (Fig. 1.4). Typically, three characteristic coppery dots can be observed on the thorax. Males can be distinguished from females by means of the non-iridescent black coloration and the short hairs on the hind-femur (Kunz 1994). Both males and females can roll up in a defensive ball posture. Adopting this position, they are completely protected against attacks from enemies, predators or theirs hosts by the hard and sculptured cuticle.

1.7.4 Behavior of female cuckoo wasps

The phenology of *H. rutilans* females is tightly linked to host appearance and lasts in central Europe from early June to September (Strohm et al. 2001). *H. rutilans* is one of the few cuckoo wasps species with a known and narrow host spectrum. Different European beewolf species (Hymenoptera, Crabronidae: *Philanthus triangulum*: Blösch 2000; Kunz 1994; Linsenmaier 1997; Strohm et al. 2001; Trautmann 1927; Veenendaal 1987, *Philanthus venustus*: Linsenmaier 1968, and *P. coronatus*: Blüthgen 1961; Kunz 1994; Morgan 1984) have been reported to be hosts of *H. rutilans*. Since *P. triangulum* is the only fairly abundant beewolf species in central Europe, *H. rutilans* is effectively monospecific in this region. A report of *Halictus zebrus* (Hymenoptera, Halictidae) as a host of *H. rutilans* seems rather questionable (Trautmann 1927).

The bees caught by the beewolves are the target of the cuckoo wasps and the developing larva will feed both on the early beewolf larva as well as the bee prey. Two oviposition strategies can be observed in *H. rutilans* when attacking *P. triangulum* (see chapter 8 for details): Females will either attack beewolf females that are trying to enter their nest with a honeybee. They try to lay their eggs on the bee while the beewolf opens the nest entrance. Alternatively, cuckoo wasps enter the beewolf nest and lay an egg onto a bee that is temporarily couched in the main burrow of the nest. In both cases, the honeybee will subsequently be brought into the brood cell. Therefore, the honeybee functions as a Trojan horse to sneak the cuckoo wasp egg into the beewolf brood cell. In the field, cuckoo wasp females can be seen in beewolf aggregations flying around and landing on beewolf nest mounds and feeding on flowers.

Fig 1.4: H*edychrum rutilans* female.

1.7.5 Behavior of male cuckoo wasps

In contrast to female behavior, knowledge about the behavior of *H. rutilans* males is largely lacking. This is the case for all cuckoo wasp species and merely no information is available about the time of mating, its location, and the incidence of male or female sex pheromones. In the field, *H. rutilans* males can often be seen sitting on flowers, preferentially on composite plants like *Erigeron annuus* (J. Kroiss, unpublished data).

1.8 OUTLINE OF THE THESIS

1.8.1 Male sex pheromones and their potential significance for female choice

In the first part of this thesis (chapters 2-6), we investigate the fascinating phenomenon of male sex pheromones and their potential significance for female mate choice with a focus on the sex pheromone of male beewolves. First of all, we describe the chemical composition of the pheromone (chapter 2) and the morphology of the PPG, the huge storage organ of the pheromone (chapter 3).

The beewolf pheromone has been suggested to play an important role for female mate choice (Herzner 2004; Herzner et al. 2006; Kaltenpoth 2006; Kaltenpoth & Strohm 2006; Schmitt 2004). In particular, it has been demonstrated that the pheromone may provide information about size (Kaltenpoth 2006) and age (Kaltenpoth & Strohm 2006) of the male, which might allow females to choose according to a model of "good genes". Furthermore it has been shown that the pheromone of brothers is more similar than that of unrelated individuals (Herzner et al. 2006). To complement these studies, we demonstrate that the composition of the multi-component male sex pheromone not only varies considerably between males of different families, but also between subpopulations, and populations across Europe and discuss the potential significance of this variation for female mate choice according a best compatibility model (chapter 4).

Thereafter, we demonstrate that the European beewolf exhibits a "hotspot" lek-polygyny as the prevailing mating system and discuss the peculiarity of this mating system in association with a male sex pheromone (chapter 5). Additionally, we discuss the special suitability of this mating system for female choice according to properties of the male sex pheromone.

In chapter 6, we describe both the chemical nature and the place of production of a newly discovered male-specific pheromone of the cuckoo wasp *Hedychrum rutilans*. This is the first description of a male-specific pheromone in cuckoo wasps and in parasitoid aculeate Hymenopterans and one of the very few reports of a male pheromone in parasitic insects.

1.8.2 The significance of CHCs for interspecific communication in a hymenopteran host-parasite system and evolutionary consequences

In the second part of this thesis, we investigate the significance of CHCs for the interspecific chemical communication in a hymenopteran host-parasite system. First, we demonstrate that females of the cuckoo wasp *H. rutilans* exploit the presence of cuticular hydrocarbons of their hosts, the European beewolf *P. triangulum*, in its nest-material for host finding (chapter 7). The presence of cuticular hydrocarbons in the nest material and its exploitation by a parasitoid has not yet been reported for a ground-nesting solitary insect.

Afterwards, we provide evidence that females of these cuckoo wasps mimic the composition of the cuticular hydrocarbons of their beewolf hosts (chapters 8 and 9). Using such a qualitative chemical mimicry, the cuckoo wasps are able to evade detection and aggression by their hosts. Furthermore, we show that the cuckoo wasps have a remarkably reduced amount of cuticular hydrocarbons compared to their hosts. We propose that this constitutes a second, quantitative, part of the cuckoo wasps' chemical strategy to avoid detection by the host in a sense of a "chemical insignificance" (chapter 10).

This reduction of the cuticular hydrocarbons might impose the problem of increased water loss to the cuckoo wasps and might require special adaptations of the cuticle to this problem. Hence, as a first step to investigate this question, we characterize the structural properties of the spectacular iridescent cuckoo wasp cuticle and investigate the proximate causes of the structural colors (chapter 11). Additionally, we discuss the potential ultimate causes for the evolution of these colors and the possible significance of the periodic nanostructures for the mechanical strength of the cuticle and thermoregulation.

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CHAPTER 2

A SELFISH FUNCTION OF A SOCIAL GLAND? A POSTPHARYNGEAL GLAND FUNCTIONS AS A SEX PHEROMONE RESERVOIR IN MALES OF THE SOLITARY WASP *PHILANTHUS TRIANGULUM*.

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2.1 SUMMARY

The postpharyngeal gland (PPG) has long been assumed to be restricted to ants, where it serves mainly functions in the maintenance of social integrity. Recently, a possibly homologous PPG has been described in females as well as in males of a solitary digger wasp, the European beewolf, *Philanthus triangulum* (Hymenoptera, Crabronidae). Female beewolves use the content of their PPG to embalm their honeybee prey in order to delay microbial growth. Here we show that in male beewolves the PPG serves yet another function. Males are territorial and scent mark their territories with large amounts of a cephalic secretion during the course of one day. We hypothesized that males store the high quantities of this marking secretion in their extremely enlarged PPG. Owing to the large size of the PPG we were able to directly suck off samples of its content for GC-MS analysis. We found 55 substances. Besides various long-chained aliphatic hydrocarbons, 13 substances with functional groups occur in the PPG. This composition is consistent with the pattern of the marking pheromone of male European beewolves described earlier. Thus, we conclude that the PPG is the storage organ of the marking pheromone. Comparisons of the PPG content and total-head extracts show a strong congruency. Thus, total-head extracts can be used for the analysis of the marking secretion of beewolves. Furthermore, we describe a dimorphism in the composition of the pheromone that is based on significant differences in the proportions of seven pheromone compounds between the two morphs.

2.2 INTRODUCTION

Hymenoptera are one of the most impressive insect orders regarding the diversity of species and levels of social organization. According to their different life styles and ecological niches, they possess a huge variety of exocrine glands with diverse functions. In solitary Hymenoptera these glands contain secretions that serve important functions in the context of mate attraction, courtship, prey paralysis, and defense (e.g. Gnatzy et al. 2004; Quicke 1997; Rathmayer 1962; Wilson 1972). Due to the increased requirements for communication and defense of social species, many of these glands have gained various new functions in bees, wasps, and ants (e.g. alarm, trail establishment, recruitment, or reproductive dominance; see Blum 1992; Hölldobler & Wilson 1990) . One of these 'social' glands, the postpharyngeal gland (PPG), has long been thought to be idiosyncratic to ants, Formicidae, and evolved in response to the requirements of the social life (e.g. Hölldobler & Wilson 1990; Lenoir et al. 1999; Schoeters & Billen 1997). The PPG of ants is a large cephalic reservoir that is involved in species (Oldham et al. 1999) and nestmate recognition (Hefetz et al. 1996; Soroker et al. 1994), as well as in task discrimination (Kaib et al. 2000). However, several other or additional functions of the PPG like digestion, caste determination or storage organ for nutrients for queens or larvae have been discussed. Not all of these roles have been well corroborated, but it can be assumed that in ants the PPG serves several functions depending on species, age, sex, caste, and mating status (Eelen et al. 2006).

Recently, a PPG has been found in females of the European Beewolf *Philanthus triangulum* (Hymenoptera, Crabronidae; formerly Sphecidae, Melo 1999), a solitary hunting wasp (Strohm et al. 2007). Based on its morphology and the chemistry of its content, the gland is considered to be homologous to the PPG known in the Formicidae (Strohm et al. 2007) . The function of the PPG in beewolf females is quite different from that in ants. Beewolf females hunt honeybee workers as prey for their offspring and store the paralyzed bees as provisions for the larvae in brood cells in the soil (Strohm 1995; Strohm & Linsenmair 1999). Females apparently apply the secretion of their PPG to the surface of their honeybee prey to delay fungal infestation in the warm and humid underground nests (Strohm & Linsenmair 2001, Herzner et al., unpublished data).

Unexpectedly, *P. triangulum* males also posses PPGs that are even much larger than in females (Herzner et al. 2007). Beewolf males establish and scent-mark territories with a marking pheromone to attract conspecific females for mating (Evans & O´Neill 1988; Schmitt et al. 2003; Simon Thomas & Poorter 1972; Strohm 1995; Strohm & Lechner 2000).

The marking pheromone of *P. triangulum* has been characterized for three different populations (Borg-Karlson & Tengö 1980; Schmidt et al. 1990; Schmitt et al. 2003) and was thought to be produced and stored in the mandibular gland (Evans & O´Neill 1988; Gwynne 1978; Schmidt et al. 1990; Schmidt et al. 1985).

However, since the mandibular gland does not have a large reservoir we hypothesized that the marking pheromone is stored in the large PPG. Therefore, we took direct samples from dissected PPG reservoirs and analyzed these by combined gas chromatography – mass spectrometry (GC-MS). We compared our findings with an earlier description of the pheromone of the same population (Schmitt et al. 2003).

The PPG content of female beewolves shows a marked dimorphism: the main component of the hydrocarbon mixture is either (*Z*)-9-pentacosene or (*Z*)-9-heptacosene (Strohm et al., unpublished data). Since these compounds also occur in the male marking pheromone and such a variation in the male pheromone might have important implications for mate choice (Herzner et al. 2006), we looked for such a dimorphism in males. Finally, we examined whether total-head extracts are sufficiently similar to samples directly taken from the PPG to allow for analyses of the pheromone by extracting heads instead of dissecting individual glands.

2.2 MATERIALS AND METHODS

2.2.1 Specimens

Adult male beewolves were obtained from a laboratory population (see e.g. Strohm & Linsenmair 1997). Freshly eclosed males were individually marked and kept as described before (Herzner et al. 2006). Since age has been shown to influence pheromone composition (Kaltenpoth & Strohm 2006) all males used for the analyses were the same age. Twelve to fourteen days after emergence, males were caught and stored individually in small polystyrene vials (35mm diameter, 82mm length, filled with 2 cm of moist sand) with rubber foam plugs for two days, so that they could replenish the reservoirs of their marking pheromone. They were then anaesthetized with CO_2 and individually frozen at -18°C until chemical analyses were conducted.

2.2.2 Extracts

For the analyses males were thawed, decapitated, and the heads dissected under a stereo microscope. The PPG was carefully uncovered by removing the frontal part of the head capsule by cutting the cuticle between the eyes, toruli, and lateral ocelli very shallowly using newly broken pieces of razorblades in a blade holder. If the reservoir was not injured it then bulges out. A sample of the PPG content was taken by inserting the tip of a fine glass pipette directly into the gland reservoir. The content was automatically sucked into the pipette by capillary forces. The sample was then dissolved in re-distilled hexane.

For the complete inventory and characterization of the content of the PPG the samples of three males were combined and reduced in volume to approximately 100 µl by a stream of nitrogen at ambient temperature. An aliquot of 1 µl was analyzed by combined gas chromatography – mass spectrometry (GC-MS) (set-up 1, manual injection). To check for any unpolar substances hidden under the peaks of polar pheromone compounds, the extract was fractionated on a conditioned SiOH-glass-column (CHROMABOND, 500mg, Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions, and 1 µl of both the hexane and the dichloromethane fraction was analyzed by combined GC-MS (set-up 1, manual injection).

Some components that could be detected and characterized in the pooled samples could not be satisfactorily identified in samples of individual males due to the smaller amounts of secretion. Therefore, the number of peaks included in the respective analyses varies somewhat.

To compare samples directly taken from the PPG with those obtained from total head extracts, 15 males were dissected as described above. After a sample of the PPG content had been taken, the remaining head capsule still containing the PPG and most of its content was extracted in re-distilled hexane for three hours. The samples were reduced in volume to approximately 100 µl and an aliquot of 1 µl of each solution was analyzed by GC-MS (set-up 1, automatic injection).

To investigate a possible dimorphism in the composition of the PPG content, we analyzed a large sample of 45 males in order to be able to detect a low frequency of one morph. Males were obtained and treated as described above. For this second analysis we did not dissect the heads, however, but extracted whole heads that were incised to open the glands. The heads were individually extracted in re-distilled hexane for three hours, the sample volume reduced

to approximately 100 µl, and an aliquot of 1µl of each sample was analyzed by GC-MS (set-up 2, automatic injection).

2.2.3 Capillary Gas chromatography – mass spectrometry. Set-up 1

GC-MS analysis was performed with an Agilent 6890N Series gas chromatograph (Agilent Technologies, Böblingen, Germany) coupled to an Agilent 5973 inert mass selective detector. The GC was equipped with an RH-5ms+ fused silica capillary column (30 m x 0.25 mm ID; df = 0.25µm), and the temperature program ramped from 60°C to 300°C with 5°C/min. The temperature was held constant in the beginning at 60°C for 1 min and at the end at 300°C for 10 min. Helium was used as a carrier gas with a constant flow of 1 ml/min. A split/splitless injector was installed at 250°C and in the splitless mode for 60 sec. The electron impact mass spectra (EI-MS) were recorded with an ionisation voltage of 70 eV, a source temperature of 230°C and an interface temperature of 315°C. The software MSD ChemStation (Agilent Technologies, Palo Alto, CA, U.S.A.) for Windows was used for data acquisition.

2.2.4 Capillary Gas chromatography – mass spectrometry. Set-up 2

GC-MS analysis was performed with a Fisons Instruments (Fisons, Egelsbach, Germany) GC 8000 Series coupled to a Fisons Instruments MD800 quadrupol mass detector. The GC was equipped with a J&W DB-5 fused silica capillary column (30 m x 0.25 mm ID; df = 0.25µm) (J&W, Folsom, CA, USA), and the temperature program ramped from 60°C to 310°C with 5°C/min. The temperature was held constant at 310°C for 10 min. Helium was used as a carrier gas with a constant pressure of 90 mbar. A split/splitless injector was set at 240°C and was in the splitless mode for 60 sec. The electron impact mass spectra (EI-MS) were recorded with an ionisation voltage of 70 eV, a source temperature of 220°C and an interface temperature of 315°C. The software Xcalibur (ThermoFinnigan, Egelsbach, Germany) for Windows was used for data acquisition.

2.2.5 Chemicals and pheromone compound identification

Solvents (Fluka, Deisendorf, Germany) were distilled and checked for purity by GC-MS prior to use. (*Z*)-9-octadecen-1-ol and 1-eicosanol were identified by comparing retention times and mass spectra of the PPG extracts with those of the synthetic standard ((*Z*)-9-octadecen-1-ol: Merck Schuchard OHG (Hohenbrunn, Germany); 1-eicosanol: Dr. Ehrensdorfer GmbH (Augsburg, Germany)). The *n*-alkanes were identified by comparing retention times and mass spectra of beewolf gland extracts with data from earlier analyses (Schmitt et al. 2003; Strohm et al., unpublished data) and with data from a commercial MS library (NIST, Gathersburg, MD, U.S.A.). The corresponding alkenes were identified by their typical mass spectra and their retention times. The positions and geometries of the double bonds were inferred by comparison with earlier analyses on the chemistry of the pheromone (Schmitt et al. 2003) and of the PPG content or cuticular hydrocarbons of conspecific females that had been characterized by FTIR spectrometry and DMDS derivatization (Strohm et al., unpublished data); peaks with identical retention times were considered to be the same isomers. Some alkenes were present only in threshold amounts in all our earlier and the current analyses and could thus not be characterized completely. The alkadienes were characterized by their typical mass spectra and their retention times. Due to their very small amounts the position and geometry of the double bonds could not be determined. Methyl alkanes were identified by diagnostic ions, standard MS databases (see above), and by determining Kovats indices by the method of (Carlson et al. 1998). The ketone Δ-16-pentacosen-8-one was identified by comparing retention time and mass spectrum of the peak with the Δ-16-pentacosen-8-one present in the female PPG (Strohm et al., unpublished data).

2.2.6 Statistics. Head extracts vs. PPG content

Peak areas were obtained by manual integration (MSD ChemStation) and relative peak areas were transformed to logcontrasts (Aitchison 1986; Reyment 1989) prior to the analysis. Mean values of each individual peak were normalized by log-transformation. To test for a chemical congruency between the substances found in the PPGs and in the head extracts, we conducted a regression analysis between the proportions of components (Aitchison- and log-transformed) in the samples directly obtained from the PPG and in the head extracts (reduced major axis regression (RMA) (Legendre & Legendre 1998) using 'RMA Software for Reduced Major Axis Regression v.1.17' (A. J. Bohonak, San Diego University, U.S.A; freely

available at http://bio.sdsu.edu/pub/andy/RMA.html). To assess the chemical similarity between the PPG samples and the head extracts, we tested whether there was a direct proportionality: i.e. the slope of the resulting regression line should not deviate significantly from 1 and the y-intercept should not deviate significantly from 0.

2.2.7 Statistics. Chemical dimorphism

Peak areas were obtained by automatic integration (Xcalibur). The relative peak areas were transformed to longcontrasts (Aitchison 1986; Reyment 1989). Since in females the dimorphism is most striking for the compounds (*Z*)-9-pentacosene and (*Z*)-9-heptacosene (Strohm et al., unpublished data), we first focused on these peaks. The different isomers of the unsaturated hydrocarbons were not well separated in the chromatograms (see below). Therefore, in the following 'pentacosene' and 'heptacosene' refer to the mixture of isomers. However, in all cases the Z-9 isomers are most dominant.

Inspection of the chromatograms revealed that based on the proportions of pentacosene and heptacosene in their pheromone blends, males could be separated into two distinct types: those with pentacosene as the by far most abundant hydrocarbon (C_{25}–type males) and those with comparable proportions of pentacosene and heptacosene (C_{25}/C_{27}–type males). To test for a bimodal distribution we analyzed the frequency distribution of the proportion of heptacosene by use of a histogram plot (see results). The histogram revealed that the males could be assigned to two groups according to their proportion of heptacosene. We compared the proportions (relative peak areas Aitchison transformed) of all substances between these two groups with *exact tests for two independent samples* (using SPSS 13.0 for Windows, SPSS Inc., Chicago, IL, U.S.A.).

2.3 RESULTS

2.3.1 PPG content

The list of substances that we found in the samples taken directly from the PPGs of three males is given in Table 1.1 (The assignment of numbers to the different compounds as given

in Table 1.1 is valid throughout the paper, including text, table, and figures.). Apart from the previously described 11 substances (Schmitt et al. 2003) we could characterize another 42 compounds, 33 of which were hydrocarbons and 9 substances with functional groups. Two new peaks could not yet be identified.

Table 1.1: Compounds detected in the postpharyngeal gland of male *P. triangulum* arranged in the order of elution on our GC-MS set-up 1.

Compound number	Compound name	Compound number	Compound name
1	Hexanoic acid	29	(Z)-9-Pentacosene
2	Nonanal	30	(Z)-7-Pentacosene
3	Octanoic acid	31	Δx-Pentacosene
4	Nonanoic acid	32	Δx-Docosenol
5	Pentadecane	33	Pentacosane
6	(S)-2,3-Dihydrofarnesoic acid	34	13-Methyl pentacosane
7	(Z)-9-Octadecen-1-ol	35	11-Methyl pentacosane
8	(Z)-10-Nonadecen-2-one	36	7-Methyl pentacosane
9	1-Octadecanol	37	(Z)-9-Hexacosene
10	Heneicosane	38	Hexacosane
11	Unidentified substance 1	39	Δ-16-Pentacosen-8-one
12	Docosane	40	Δx,y-Heptacosadiene
13	(Z)-11-Eicosen-1-ol	41	(Z)-9-Heptacosene
14	Δx,y-Tricosadiene	42	(Z)-7-Heptacosene
15	(Z)-9-Tricosene	43	Heptacosane
16	(Z)-7-Tricosene	44	13-Methyl heptacosane
17	1-Eicosanol	45	11-Methyl heptacosane
18	Δx-Tricosene	46	Octacosane
19	Tricosane	47	Δ-18-Heptacosen-10-one
20	5-Methyl tricosane	48	(Z)-9-Nonacosene
21	7-Mehtyl tricosane	49	Nonacosane
22	9-Methyl tricosane	50	15-Methyl nonacosane
23	Δx,y-Tetracosadiene	51	13-Methyl nonacosane
24	(Z)-9-Tetracosene	52	Triacontan
25	(Z)-7-Tetracosene	53	(Z)-9-Hentriacontene
26	Tetracosane	54	(Z)-7-Hentriacontene
27	Unidentified substance 2	55	Hentriacontane
28	Δx,y-Pentacosadiene		

2.3.2 Head extracts vs . PPG content

In the samples directly taken from the PPG and in the head extracts of the 15 individual males we were able to detect 30 compounds. Several substances had to be combined for the analysis, since peaks were not always clearly separated (Fig. 2.1). A total of 20 peaks (Aitchison- and log-transformed) could be subjected to the correlation and regression

analyses. All substances found in the PPG samples were also present in the total-head extracts and *vice versa*. Furthermore, the relative amounts of the substances in the heads showed a strong linear correlation with the corresponding substances in the PPG that did not deviate significantly from direct proportionality (Fig. 2.1) (slope of the RMA-regression line: 0.960 (95% confidence intervals: 0.797 - 1.123), y-intercept -0.1247 (95% confidence intervals: -0.23911 - -0.01037)). The only substance deviating conspicuously from the regression line was (*S*)-2,3-dihydrofarnesoic acid, which was always more abundant in the head extracts.

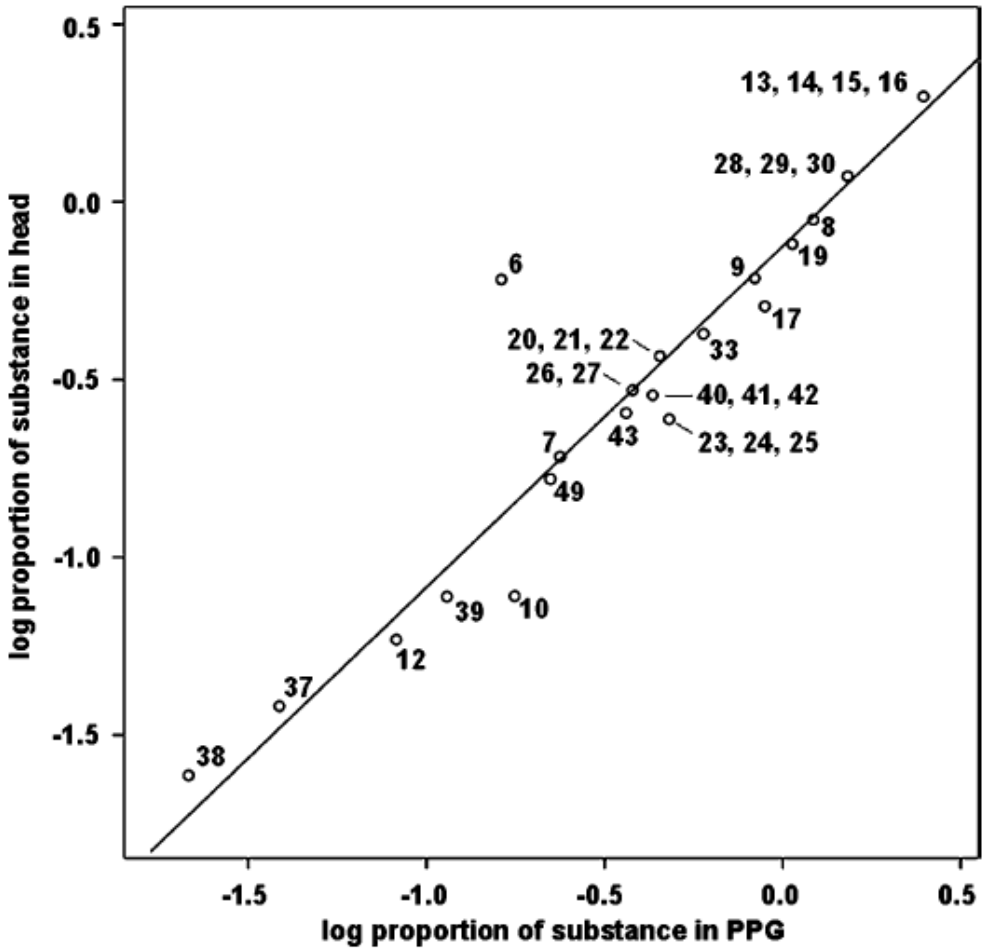

Fig. 2.1: Correlation between the relative amount (relative peak area Aitchison and log transformed, see text) of a particular component in the postpharyngeal gland (PPG) and in head extracts of males of the European beewolf ($r^2 = 0.88$, N = 20 compounds, $P < 0.001$). The regression line was generated using reduced major axis regression ($y = 0.9600x - 0.1247$). The data are based on extracts of 15 males. The numbers correspond to the numbers in the peak list (Table 1.1).

2.3.3 Chemical dimorphism

23 peaks could be reliably identified in all 45 samples and were included in this analysis. As above, several peaks had to be combined for the analysis (Fig. 2.4). The chromatograms and the histogram revealed that among the 45 males analyzed 39 individuals (87%) belonged to the C_{25}-type with pentacosene as the predominant hydrocarbon, whereas six individuals belonged to the C_{25}/C_{27}-type with pentacosene and heptacosene in approximately equal proportions (Fig. 2.2 and 2.3). Besides the differences in the proportions of pentacosene and heptacosene the two types significantly differed in five other peaks (Fig. 2.4).

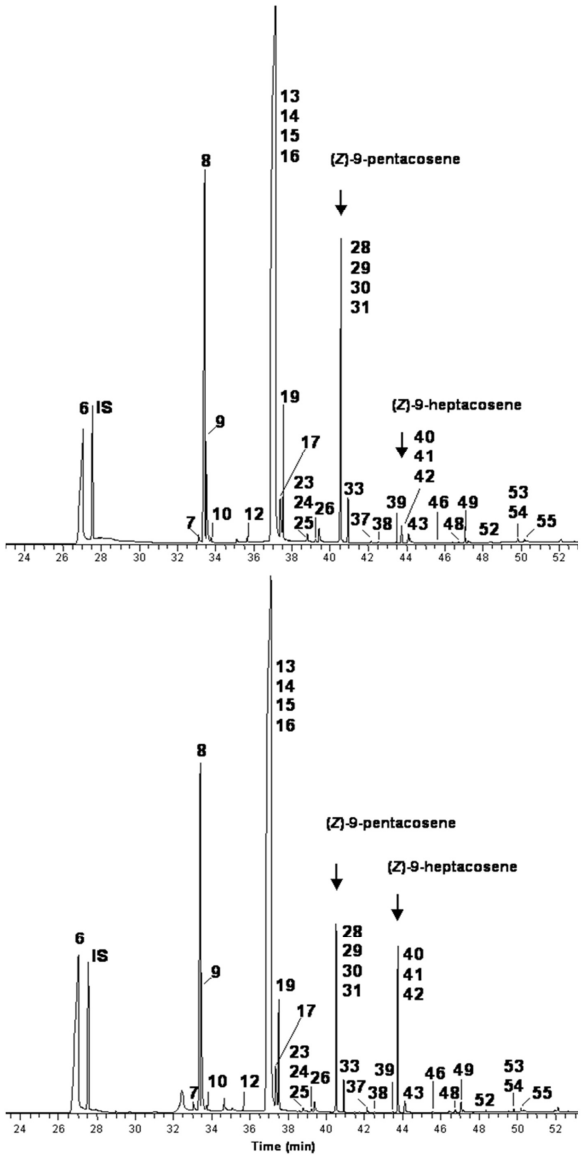

Fig. 2.2: Total ion chromatograms of the head extracts of an individual male with pentacosene as the predominant hydrocarbon peak (C_{25}-type, A) and an individual male with similar proportions of pentacosene and heptacosene (C_{25}/C_{27}-type, B). The numbers correspond to the numbers in the peak list (Table 2.1). IS: Internal Standard (octadecane).

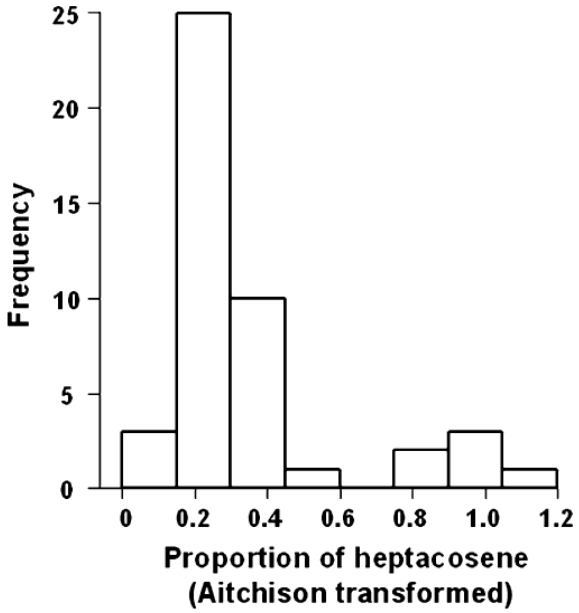

Fig. 2.3: Frequency distribution of the proportion of heptacosene (mixture of isomers, see text) in the head extracts of 45 males. The males can clearly be separated into two groups with no overlap.

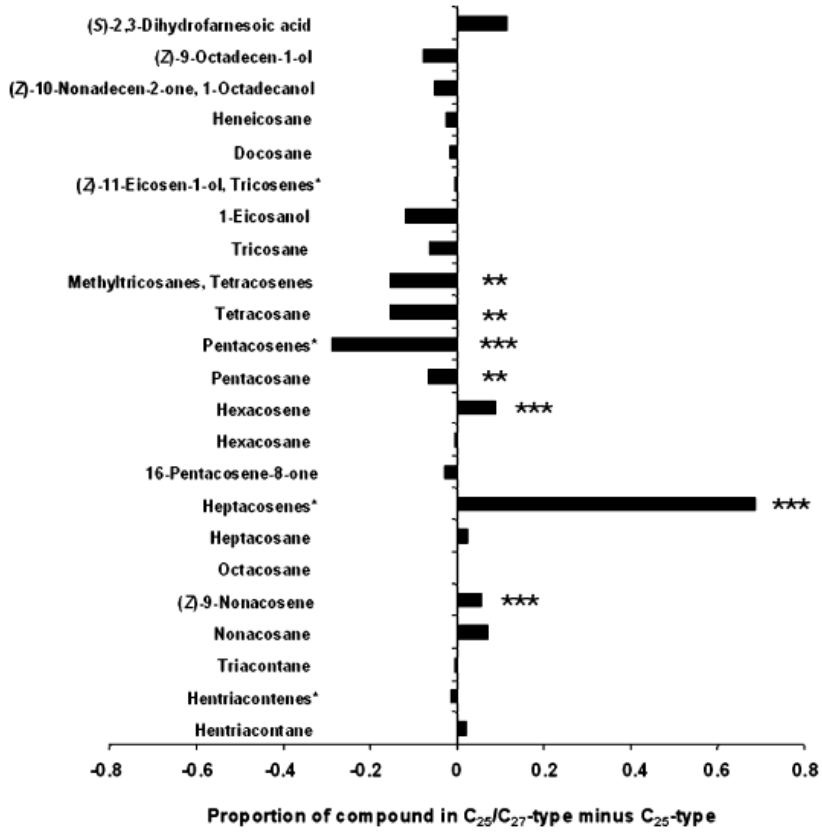

Fig. 2.4: Differences between the C_{25}-type and the C_{25}/C_{27}-type in the mean proportions of individual components of the head extracts (Aitchison-transformed, N = 45 males). Bars to the right of the line (positive values) indicate that the proportion of the given substance is higher in the C_{25}/C_{27}-type, bars to the left of the line (negative values) indicate that the proportion of the substance is higher in the C_{25}-type. The asterisks indicate significant differences (exact tests for independent samples; ***: $P < 0.0001$; **: $P < 0.001$).

2.4 DISCUSSION

Our chemical analyses of samples directly taken from the PPG of male *P. triangulum* revealed a blend of 55 substances including 11 compounds previously described as components of the marking pheromone in *P. triangulum* males (Schmidt et al. 1990; Schmitt et al. 2003). These results, together with our observation that (as described for other *Philanthus* species (Schmidt et al. 1985)) the compounds isolated from heads or PPGs of male *P. triangulum* can also be found on freshly marked territories of beewolf males (Herzner, Kaltenpoth, Kroiss, Schmitt, and Strohm, unpublished data), support our hypothesis that the PPG of male European beewolves functions as the reservoir of the marking pheromone.
The 44 substances that had not been described by (Schmitt et al. 2003) were included here due to different reasons. In the earlier study most of the minor hydrocarbons were omitted, because they were also found in cuticle extracts and we concluded that they might not be part of the marking pheromone (see also Schmidt et al. 1990). Here we show that the hydrocarbons are in fact present in the PPG. Therefore, we consider them as a part of the marking secretion. Most importantly, for the current study we pooled the gland extracts of three large males, concentrated the resulting sample considerably and injected it by hand. This procedure allowed for the detection of very minor substances in the pheromone blend.

One of the compounds that is new to the cephalic secretion of male European beewolves is (*Z*)-9-octadecen-1-ol. It has been previously isolated from glands and cuticle of several apid species (see e.g. Andersen et al. 1988; Bergström 1985) and from jojoba (*Simmondsia chinensis*) seeds (Tobares et al. 2003). 1-Eicosanol has been described as a component of the marking secretion of males of a cuckoo bumblebee (Kullenberg et al. 1970) and is present in the female sex pheromone of the European grapevine moth *Lobesia botrana* (Arn et al. 1988). It can also be found on cocoons and on the cuticles of different developmental stages of the honeybee *Apis mellifera* (Donzé et al. 1998). The ketone Δ-16-pentacosen-8-one is also present in the PPG of *P. triangulum* females (Strohm et al., unpublished data). Δ-18-peptacosen-10-one has been found to be a pheromone component of the white-spotted longicorn beetle *Anoplophora malasiaca* (Yasui et al. 2003).

Based on descriptions in the literature (Borg-Karlson & Tengö 1980; Evans & O´Neill 1988; Gwynne 1978; McDaniel et al. 1992; Schmidt et al. 1990; Schmidt et al. 1985) we had initially assumed that the marking pheromone in *P. triangulum* is secreted from the mandibular glands. Owing to its delicate structure (Herzner et al. 2007) the PPG of male beewolves is

destroyed when dissecting heads to remove and extract the mandibular glands. When the PPG is injured, its content instantly spills out and disperses in the head capsule. As a consequence the mandibular gland gets contaminated with the PPG content. This was possibly the case in some previous studies on beewolf male pheromones (Borg-Karlson & Tengö 1980; Schmidt et al. 1990; Schmidt et al. 1985; Schmitt et al. 2003).

When taking extreme care during dissection it is possible to remove the mandibular glands without harming the PPG. When we extract these mandibular glands that are not contaminated with the content of the PPG we mostly detect only very minor amounts of some pheromone components in the GC-MS (Herzner & Strohm, unpublished data). Therefore, in total head extracts, the vast majority of the detected compounds seems to originate from the PPG and not from the mandibular glands.

In our analyses all components of the PPG were also present in total head extracts and *vice versa*. Furthermore, the proportions of all compounds were very similar between the two types of extracts. The only component that showed a marked discrepancy in its relative abundance between the PPG and the head extracts was (*S*)-2,3-dihydrofarnesoic acid, which was always more abundant in the head extracts. The reason for this difference is yet unclear. Owing to the extraordinary size of the PPG (Herzner et al. 2007), its content dominates head extracts in a way that the contribution of other tissues or the cuticle to the extract can be neglected. The high congruence between direct samples of the PPG and total head extracts of male European beewolves shows that the latter yield valid samples of the marking secretion. Therefore, it seems justified and far more practical to use total-head extracts for studies on the male beewolf marking pheromone. In particular, when doing quantitative analyses the risk of loosing some secretion due to non perfect dissection can be circumvented.

The PPG has long been considered to be idiosyncratic to ants. In ant workers it typically contains a blend of straight and methyl-branched hydrocarbons (Cabrera et al. 2004; Lucas et al. 2004; Soroker et al. 1995) that mainly plays a role in nestmate recognition (Crozier & Dix 1979; Lenoir et al. 1999; Soroker et al. 1998; Vienne et al. 1995). Female *P. triangulum* also possess large PPGs (Strohm et al. 2007) that contain a blend of mainly straight unsaturated and saturated as well as minor proportions of methyl-branched long-chain hydrocarbons and long-chain unsaturated ketones (Strohm et al., unpublished data). Many of the hydrocarbons as well as the ketones Δ-16-pentacosen-8-one and Δ-18-heptacosen-10-one present in the male PPG are also found in the female PPG. The female PPG is morphologically different from the male gland and contains, besides the ketones, no substances with functional groups. The PPG secretion of female beewolves is used for prey

preservation (Strohm & Linsenmair 2001) and thus has a completely different function from the PPG content in beewolf males.
Thus, in beewolf females the PPG is subject to natural selection to increase the protection against fungal infestations of their honeybee prey and larvae (Strohm & Linsenmair 2001). The PPG of males, by contrast, is subject to sexual selection (Herzner 2004; Herzner et al. 2006; Herzner et al. 2005; Kaltenpoth & Strohm 2006). Such a change in function and sexual dimorphism regarding glands and their contents is not unusual (e.g. the methathoric and dorsal abdominal glands in some Heteroptera (Aldrich 1988; Aldrich et al. 1996; Ho et al. 2003; Zhang & Aldrich 2003)). The significance of this sexual dimorphism remains mostly unclear, however. Even in ants, the morphology and function of the PPG seem to exhibit inter- and intraspecific variability (Eelen et al. 2006; Schoeters & Billen 1997).

In addition to the morphological and chemical differences of the PPG between the sexes there is a dimorphism with regard to the composition of the gland content within each sex of *P. triangulum*. The majority of males (87%) had pentacosene as the by far most abundant hydrocarbon (C_{25}-type). Some males (13%), however, possessed almost equal proportions of pentacosene and heptacosene (C_{25}/C_{27}-type) with a bimodal and non overlapping distribution of the two types with regard to the proportion of heptacosene. The chemical dimorphism in the PPG content of beewolf females is much more pronounced than in males. In females, the main compound, accounting for 70-80% of the blend, is either (*Z*)-9-pentacosene or (*Z*)-9-heptacosene and there are no intermediate types with similar amounts of both compounds like the C_{25}/C_{27} type in males (Strohm et al., unpublished data). The proportions of the pentacosene-morph and the heptacosene-morph in females are 80% and 20% and thus similar to the proportions of the C_{25}-type and the C_{25}/C_{27}-type in males (87% vs. 13%). The meaning of this chemical dimorphism is not yet clear. Noteworthy, the dimorphism is only exhibited by hydrocarbons without functional groups. Moreover, if the dimorphism had a genetic basis, recognition of relatives - in particular with regard to the avoidance of inbreeding (Herzner et al. 2006) – would be facilitated.

Our findings might have important implications for the evolution of the PPG within the Hymenoptera. If the PPG existed only in ants, it would be tempting to assume that it had evolved in response to the requirements of the social communication system (Eelen et al. 2006; Hölldobler & Wilson 1990; Lenoir et al. 1999). The existence of a PPG in beewolf females suggested that this gland might have evolved in some early Aculeates for the purpose of prey preservation (Strohm & Linsenmair 2001, Strohm et al., unpublished data). Now we know that the PPG in male beewolves stores the marking pheromone. This beewolf marking pheromone most probably represents the male sex pheromone, since females are obviously attracted to the territories by the windborne pheromone and most matings occur in the

males' territory or in the nearby vegetation (Alcock 1975; Evans & O´Neill 1988; Gwynne 1978; Herzner et al. 2006; Herzner et al. 2005; O'Neill 1979; O'Neill 1983; Schmitt et al. 2003; Simon Thomas & Poorter 1972; Strohm 1995; Strohm & Lechner 2000). Therefore, the pheromone is subject to sexual selection with regard to both its composition and amount (Herzner 2004; Herzner et al. 2007; Herzner et al. 2006; Herzner et al. 2005; Kaltenpoth & Strohm 2006; Herzner et al., unpublished data). Since sexual selection can be a very strong evolutionary force (Higashi et al. 1999; Kaneshiro & Boake 1987; Lande 1981) the evolution of the gland might have been influenced by the requirements of males to store large amounts of pheromones to attract females. Regardless of the evolutionary origin of the PPG our findings show that this gland, that was assumed to have a 'social' function in ants, serves a selfish function as a reservoir for a male sex pheromone in a solitary wasp.

2.5 ACKNOWLEDGEMENTS

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CHAPTER 3

MALES OF A SOLITARY WASP POSSESS A POSTPHARYNGEAL GLAND.

Arthropod Structure and Development 36: 123-133

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3.1 SUMMARY

The postpharyngeal gland has long been thought to occur only in ants. Here we characterize, by use of light and electron microscopy as well as 3D reconstruction based on nuclear magnetic resonance (NMR) imaging data, a large cephalic gland reservoir of males of a solitary digger wasp, the European beewolf, *Philanthus triangulum*. Several lines of evidence suggest that this reservoir is a postpharyngeal gland. The gland reservoir originates from the posterior part of the pharynx and consists of two pairs of unbranched tubular structures that occupy a large portion of the head capsule. Its wall is composed of a unicellular epithelium that is lined by a cuticle. The gland contains a blend of hydrocarbons and compounds with functional groups, and we show that the hydrocarbon fraction of the pheromone is congruent with the hydrocarbons on the cuticle. We discuss the implications of our findings for the evolution of the postpharyngeal gland in ants.

3.2 INTRODUCTION

The postpharyngeal gland (PPG) has long been thought to be idiosyncratic to the Formicidae (Hölldobler & Wilson 1990; Jackson & Morgan 1993; Lenoir et al. 1999; Schoeters & Billen 1997; Soroker et al. 1995), where it is involved in the formation and distribution of a nest-specific chemical signature (the 'gestalt odor'; Crozier & Dix 1979) that mediates nestmate recognition (Hefetz et al. 1992; Lenoir et al. 1999; Soroker et al. 1998; Soroker et al. 1995; Soroker et al. 1995; Soroker et al. 1994; Vienne et al. 1995). It usually contains a multi-component blend of hydrocarbons (HCs) (Cabrera et al. 2004; Hefetz et al. 1992; Lucas et al. 2004; Soroker et al. 1995; Vander Meer et al. 1982) that shows a high chemical congruency with the epicuticular chemical profile (Bagneres & Morgan 1991; Do Nascimento et al. 1993; Lucas et al. 2004; Soroker et al. 1995). In ant queens the PPG may contain some kind of queen pheromone (Dietemann et al. 2003; Vargo & Hulsey 2000). For a review of additional hypothesis on the function of the PPG in ants, see (Eelen et al. 2006).

Recently, a PPG was found in females of a solitary hunting wasp, the European beewolf *Philanthus triangulum* F. (Hymenoptera, Crabronidae; formerly Sphecidae) (Strohm et al. 2007). While the structure, location, and chemical content of the PPG of beewolf females are similar to those of ants (Strohm et al. 2007 E. Strohm, G. Herzner, T. Schmitt, unpublished), its function is very distinct. Female *P. triangulum* hunt honeybees as food for their larvae and store the paralyzed bees in their subterranean nests (Herzner et al. 2005; Strohm 1995; Strohm 2000; Strohm & Linsenmair 1999; Strohm & Linsenmair 2000). To preserve their prey from microbial degradation, beewolf females apply the secretion of their PPG to the surface of their prey (Herzner et al., unpublished). The PPG secretion prevents fungus growth on the larval provisions (Strohm & Linsenmair 2001) and in this way enhances the survival probabilities of the offspring.

It is known that PPGs also occur in male ants (Phillips Jr. & Vinson 1980). Therefore we investigated males of the European beewolf in order to extend the knowledge on the distribution and possible functional variety of the PPG among Hymenoptera. Male beewolves establish and scent mark territories to attract conspecific females (Evans & O'Neill 1988; Schmitt et al. 2003; Simon Thomas & Poorter 1972; Strohm 1995; Strohm & Lechner 2000). In an accompanying study we show that this marking pheromone is stored in an extraordinary large cephalic reservoir (Kroiss et al. 2006). The aim of the current study is to assess by virtue of morphological and chemical analyses, whether this reservoir for the marking pheromone of male *P. triangulum* is a PPG.

To qualify as a PPG the reservoir has to meet the following criteria. First, its location should correspond to the PPG of ants and beewolf females, i.e. it should extend from the posterior part of the pharynx along the proximal spines of the suspensorium. Second, the wall of the reservoir should be formed by a unicellular epithelium and lined by a cuticle (Peregrine et al. 1973; Schoeters & Billen 1997; Soroker et al. 1995). Third, in female *P. triangulum*, as in ants, the substances that are present in the PPG also comprise the cuticular chemical profile of the animals (E. Strohm, G. Herzner, T. Schmitt, unpublished). To test for a similar chemical congruency in male *P. triangulum* we compared the chemical profiles of the PPG content and the cuticle.

3.3 MATERIALS AND METHODS

3.3.1 PPG morphology

3.3.1.1 Specimens/histological investigation

Adult male beewolves were obtained from a field population in Würzburg or from a laboratory population (see e.g. Strohm & Linsenmair 1997). They were anaesthetized with CO2 and decapitated. The heads were then dissected in cold physiological saline (130 mM NaCl/5 mM KCl/4 mM MgCl2/5 mM CaCl2/15 mM HEPES/25 mM glucose/160 mM sucrose, pH 7.2) under a stereomicroscope. The first cut was performed horizontally close to the toruli. The second and third cuts were made vertically just medial to the eyes. The last cut was made horizontally from eye to eye between the mid ocellus and the lateral ocelli. The greatest care was taken to cut only through the cuticle but no subjacent structures. The cuticular flap and the subjacent air sacs were then carefully removed. The cuticular opening was carefully enlarged step by step so that finally the delicate glands could be removed from the head unharmed. The glands were then transferred onto microscope slides, immersed in physiological saline and examined under stereo- and compound microscopes.

Histological investigations of male heads were conducted using light microscopy following standard histological methods (see e.g. Strohm & Linsenmair 1995). In brief, heads were fixed in alcoholic Bouin, then rinsed in 70% ethanol, dehydrated in a graded ethanol series and propylene oxide and embedded in Durcupan (Fluka, Deisenhofen, Germany). To enable the embedding medium to soak into the head capsules of the specimens, one or both eyes

were cut off from the head capsule using razor blades. Semithin sections (4 mm) were cut on a steel-blade microtome and stained with Methylene-Blue-Azur II (Böck 1989). Specimens were viewed under a Zeiss Axioskop. Due to the partly low contrast of some delicate structures despite staining, we used phase contrast as well as differential interference contrast. Photographs were taken using a Zeiss AxioCam HRc digital camera and Zeiss AxioCam software (Carl Zeiss, Germany).

3.3.1.2 Scanning Electron Microscopy

For scanning electron microscopy (SEM), specimens were fixed in alcoholic Bouin for 3 h, washed in 70% ethanol twice, and dehydrated in a graded acetone series. The objects were then critical point dried (BAL-TEC CPD 030), sputtered with Pt/Pd (BAL-TEC SCD 005) and examined through a digital Zeiss DSM 962.

3.3.1.3 Transmission Electron Microscopy

Objects for transmission electron microscopy (TEM) were fixed overnight at 4 °C in a solution of 2.5% glutardialdehyde and 2% formaldehyde in Sörensen phosphate buffer. After postfixation in 2% OsO_4 in Sörensen phosphate buffer and dehydration in a graded ethanol series, the specimens were embedded in Epon 812. The ultrathin sections of about 70 nm thickness (Reichert Ultracut E microtome/45° diamond knife) were stained with 2% uranyl acetate and Reynold's lead citrate. The sections were examined with a Zeiss EM10 at 80 kV. Unfortunately, the fine structure of the cells of the epithelium that forms the wall of the reservoir was not optimally conserved in all regions of the reservoir, although – besides the ones described – we tried several different fixatives and conditions during fixation. Thus, some very fine structures might have been lost. We nevertheless provide the results here, since some important evidence could be gained.

3.3.1.4 Nuclear magnetic resonance imaging and 3D reconstruction

Since the dissections did not fully reveal the position of the glands in the head, we reconstructed the cephalic structures in the head by use of nuclear magnetic resonance (NMR) imaging. A male beewolf (head width 3.3 mm) was anaesthetized with CO2 and killed with diethyl ether. After decapitation the head was immersed in 100% ethanol and kept for 1 h in an exsiccator with water jet vacuum. In this manner the air sacs were filled with liquid so that air could not cause susceptibility artifacts in the imaging experiment. The head was

transferred to a 5 mm NMR tube filled with Gadovist (5 mM) (Schering, Berlin, Germany) as a contrast agent and evacuated for another 30 min. NMR imaging was carried out on a 17.6 T (750 MHz) widebore magnet using AVANCE console, Micro2.5 microimaging gradients capable of 1 T/m maximum gradient strength and a 5 mm birdcage cole (Bruker Analytic, Rheinstetten, Germany). Three-dimensional data sets were obtained using a 3D FLASH sequence. Data acquisition parameters were TR 40 ms, TE 3.0 ms, number of averages 6, a data matrix of 256 x 256 x 256 points and an isotropic spatial resolution of $(20\ \mu m)^3$. Total data acquisition time was 4.4 h. 3D reconstruction based on the NMR data was conducted using the 3D visualization software AMIRA (Indeed-Visual Concepts, Berlin, Germany). Different structures in the head were manually marked with different colors, so that finally these structures could be visualized in their natural arrangement. The results were in perfect accordance with the structure that could be inferred from the dissections and from histological analyses. Thus, there is no evidence for any significant artifact caused by the treatment or the NMR experiment. The volumes of the head capsule and of the different parts of the gland reservoir were determined in the reconstructed head.

3.3.2 Chemical congruency between PPG content and cuticle

3.3.2.1 Extracts

Newly emerged males of our laboratory population were individually marked and released into an environmental chamber (240 x 180 x 210 cm; 26/22 °C day/night and 14 h/10 h light/dark cycle) containing sand-filled buckets for nesting and artificial territories. The animals were provided with honey *ad libitum*. These conditions induce males to establish and scent mark territories (Strohm 1995). Since pheromone composition is age dependent (Kaltenpoth & Strohm 2006), all males used for the analyses were about the same age. Twelve to 14 days after emergence, males were caught and stored individually in small polystyrene vials (35 mm diameter, 82 mm length, filled with 2 cm moist sand) with rubber foam plugs for 2 days, so that they could replenish their glands. They were then individually frozen at -18 °C.

For chemical analyses, 14 males were thawed, their heads were cut off, affixed by an insect needle and dissected under a stereo microscope as described above (but without the saline). Dissection was carried out on sheets of filter paper that were renewed for each male. Instead of removing the gland from the head, we took a sample of the pure PPG content by inserting a fine Pasteur pipette directly into the gland. The secretion was automatically sucked into the

pipette by capillary forces. The sample was then dissolved in re-distilled hexane. All dissection instruments were cleaned in re-distilled hexane prior to the handling of the next specimen. The remaining thoraces and abdomens were (both parts combined but for each male individually) extracted in re-distilled hexane for 5 min (surface washes). The volumes of the extracts were reduced to approximately 100 µl by a stream of nitrogen at ambient temperature. An aliquot of 1 µl of each sample was analyzed by combined gas chromatography – mass spectrometry.

3.3.2.2 Gas chromatography – mass spectrometry

GC-MS analysis was performed with an Agilent 6890N Series gas chromatograph (Agilent Technologies, Böblingen, Germany) coupled to an Agilent 5973 inert mass selective detector. The GC was equipped with an RH-5ms+ fused silica capillary column (30 m x 0.25 mm ID; df = 0.25 µm), and the temperature program ramped from 60 °C to 300 °C with 5 °C/min. The temperature was held constant in the beginning at 60 °C for 1 min and at the end at 300 °C for 10 min. Helium was used as carrier gas with a constant flow of 1 ml/min. A split/splitless injector was installed at 250 °C and in the splitless mode for 60 s. The electron impact mass spectra (EI-MS) were recorded with an ionization voltage of 70 eV, a source temperature of 230 °C and an interface temperature of 315 °C. The software MSD ChemStation (Agilent Technologies, Palo Alto, CA, USA) for Windows was used for data acquisition.

The identification of the PPG content and the cuticular substances was accomplished by comparing retention times and mass spectra with data from earlier analyses (Kroiss et al. 2006; Schmitt et al. 2003 E. Strohm, G. Herzner, T. Schmitt, unpublished) and with data from a commercial library (NIST, Gathersburg, MD, USA).

3.3.2.3 Statistics

Peak areas were obtained by manual integration and the total peak area of each individual extract was standardized to 100%. Because relative peak areas represent compositional data, the areas were transformed to log-contrasts (Aitchison 1986; Reyment 1989) prior to analysis. The means of all peaks for the 14 individual males were calculated and subsequently normalized by log-transformation to allow for parametric testing. To test for a chemical congruency between the HCs found in the gland reservoirs and on the cuticles of beewolf males, we conducted a correlation and regression analysis between the mean proportions of components (Aitchison- and log-transformed) in the reservoir and on the cuticle. Since both

variables are GC-MS measurements and thus have the same measurement error we used a reduced major axis regression to describe their relationship (Legendre & Legendre 1998) using RMA Software for Reduced Major Axis Regression v.1.17 (A.J. Bohonak, San Diego State University, USA; freely available at http://www.bio.sdsu.edu/pub/andy/RMA.html). To assess the chemical similarity between the reservoir and the cuticle, we tested whether there was a direct proportionality: i.e. the slope of the resulting regression line should not deviate significantly from 1 and the y-intercept should not deviate significantly from 0.

3.3 RESULTS

3.3.1 Morphology

Dissection of the heads revealed that males of the European beewolf possess, besides the already known mandibular gland (Evans & O´Neill 1988 E. Strohm and W. Goettler, unpublished), a large multi-compartment gland reservoir. The reservoir consists of two pairs of tube-like evaginations of the pharynx that branch off the pharynx at the posterior part of the hypopharyngeal plate (Fig. 3.1). The upper pair of evaginations extends dorsally subjacent to the frontal cuticle of the head, winds around the brain to proceed ventrally, again subjacent to the cuticle, and ends slightly above the base of the mandibles. The basal parts of these tubes are supported by the suspensorium. The lower pair of evaginations extends slightly ventrally and then proximally towards the rear end of the head capsule and is considerably smaller. The walls of the gland reservoir appear to be very thin and translucent and are very easily damaged during dissection. The content of the reservoir is clear and oily.

The semithin sections and the 3D reconstruction based on the NMR data confirmed that the reservoir has two main parts (Fig. 3.2). The larger (longer) part originates above the pharynx, virtually embraces the brain and takes up a considerable amount of space within the head capsule (2.12 µl in a male with 3.3 mm head width and 9.3 µl head capsule volume). The lower part (0.41 µl) originates below the pharynx and extends straight to the rear wall of the head capsule. The combined volume (2.53 µl) makes up about one-quarter of the head capsule volume.

Light microscopy of semi-thin sections (Fig. 3.3) showed that the gland reservoir is formed by a monolayered epithelium. The cells of the epithelium are of an irregular more or less triangular shape with the tip pointing towards the lumen. These cells bear long hairs that

range into the lumen (Fig. 3.3A). There are no ducts of class 3 gland cells (classification after Noirot & Quennedey 1974) opening into the lumen although in the immediate vicinity of the walls of the reservoir there are numerous acini of class 3 gland cells that belong to the mandibular gland (Fig. 3.3A, E. Strohm and W. Goettler, unpublished). The nuclei are small compared to those of the gland cells of the mandibular gland. There are no visible remains of the gland content in the semithin sections, the lumen looks entirely clear. The lower and upper parts of the gland reservoir look basically very similar in all these respects.

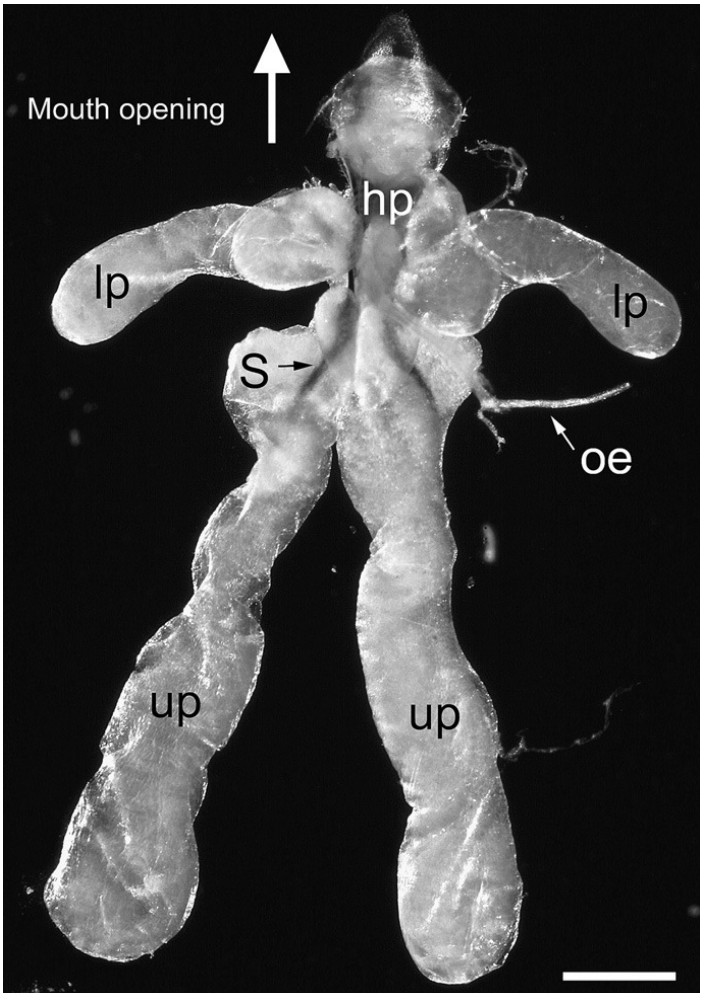

Fig. 3.1: Dissected postpharyngeal gland of a male *P. triangulum*. The two pairs of symmetrical tubes are clearly visible (scale bar 0.5 mm; the corresponding male had a head capsule width of 3.1 mm). hp, hypopharyngeal plate; lp, lower part of PPG; oe, oesophagus; S, suspensorium (shining through the gland); up, upper part of PPG.

Both parts of the reservoir show paired openings to the pharynx at the cuticular spines that form the suspensorium (Fig. 3.3B-D). The openings of the upper part are located more proximally than those of the lower part. The interconnection between the gland and the alimentary tract entails some advantages for the delivery of the secretion but also requires

specialized mechanisms to control the routes of secretion and food. Male *P. triangulum* apply the secretion from the reservoir, i.e. their marking pheromone, to the territories with a clypeal brush (Evans & O´Neill 1988 E. Strohm, G. Herzner, J. Kroiss, unpublished). The pheromone from the reservoir can easily reach this brush through the pharynx and the mouth opening. The intake of food into the gland has to be impeded, however. In the semi-thin sections we found two different types of closing flaps that might control the flow of secretion and food. All openings of the gland into the pharynx have a similar basic organization. Each one is covered by a flattened multicellular flap (reservoir closing flaps; Fig. 3.3B-D). The flaps are fixed at their distal sides and not attached at their proximal sides and we propose that they cover the gland reservoir openings when food is swallowed. This closing mechanism could thus prevent the uptake of food into the reservoirs.

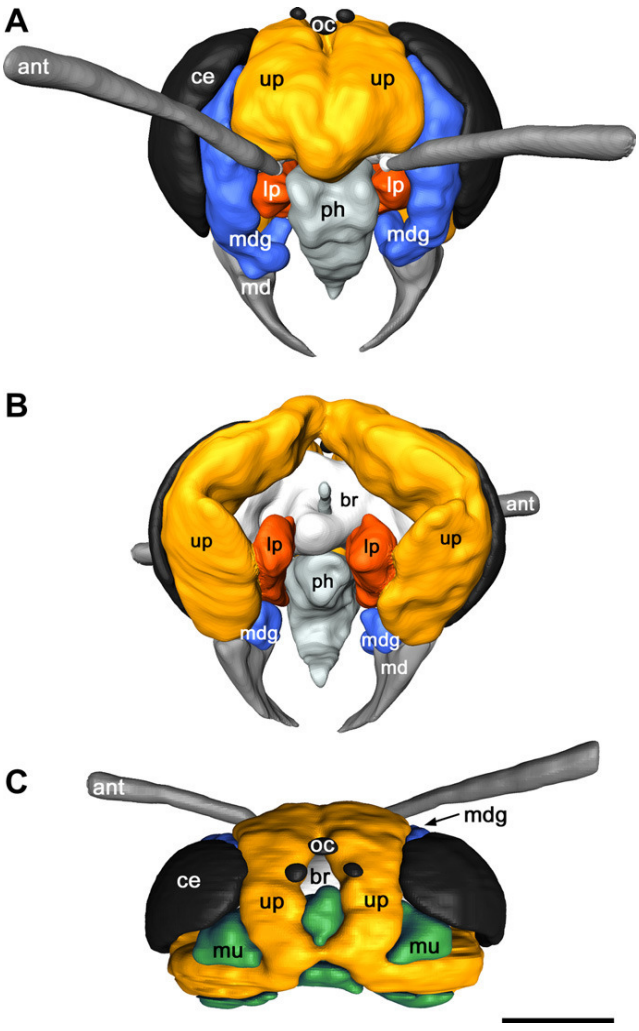

Fig. 3.2: 3D reconstruction of the postpharyngeal gland of a male *P. triangulum*. (A) Frontal view. (B) Rear view. (C) Top view (scale bar 1 mm). Please note that all four tubes of the PPG are entirely separated from each other and not connected as might appear to be the case due to the constraints of contrast and resolution of the illustration. ant, antenna; br, brain; ce, compound eye; lp, lower part of PPG; md, mandible; mdg, mandibular gland; mu, mandibular muscles; oc, ocellus; ph, pharynx; up, upper part of PPG.

How the reservoirs are opened and their content released is less clear yet. In the flap covering the opening of the lower part small muscle fibers are visible (Fig. 3.3B). These might be able to retract the flap and open the reservoir. A simultaneous contraction of other muscles or an increase of the hemolymph pressure in the head might then press out the content.

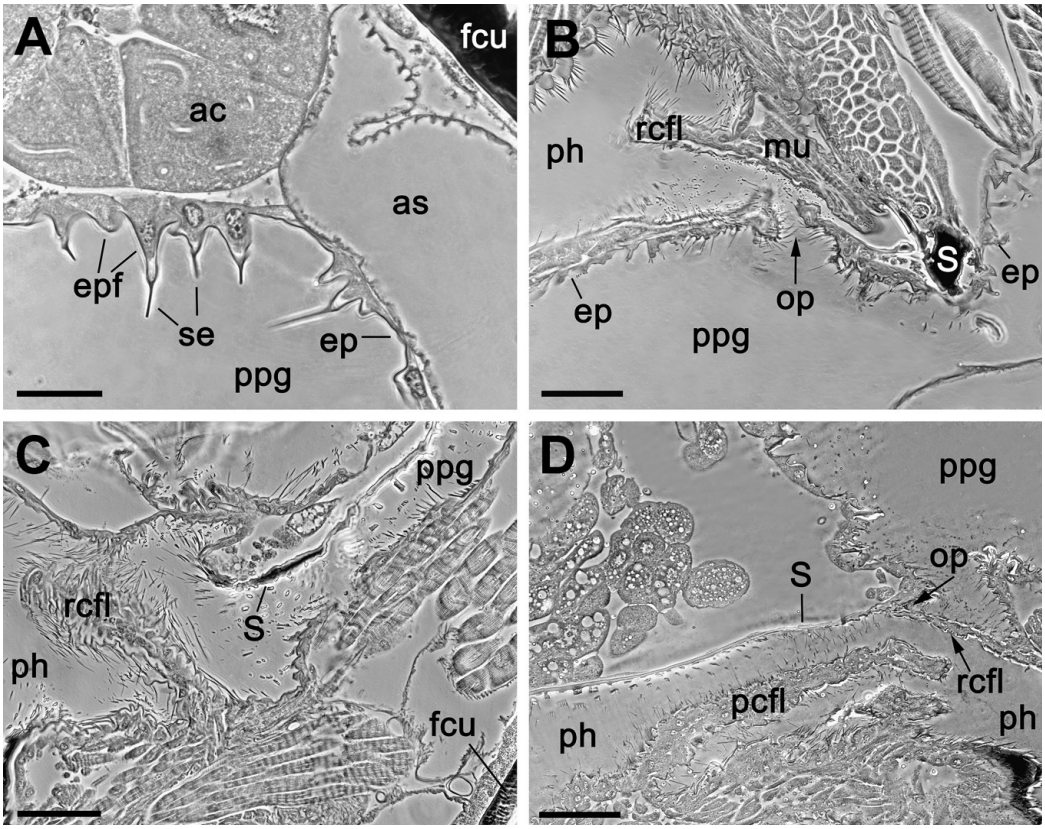

Fig. 3.3: Semithin sagittal (A-C) and cross sections (D) through the head of a beewolf male. (A) Postpharyngeal gland with air sac and an acinus that belongs to the adjacent mandibular gland (scale bar 20 µm). (B) Opening of the lower part of the PPG with reservoir closing flap; mouth opening is on the right side (scale bar 50 µm). (C) Opening of the upper part of the PPG with reservoir closing flap; mouth opening is on the bottom side (scale bar 50 µm). (D) Opening of the lower part of the PPG with pharynx closing flap and PPG reservoir closing flap; mouth opening is on the right side (scale bar 50 µm). See text for details. ac, acinus of the mandibular gland; as, air sac; ep, epithelium of the PPG; epf, epithelial folds; fcu, frontal (facial) cuticle; se, hairs; mu, muscles; op, opening of the PPG; pcfl, pharynx closing flap; ph, pharynx; ppg, lumen of the PPG; rcfl, (PPG) reservoir closing flap; S, suspensorium.

In cross sections a second set of flaps with similar structure is visible (Fig. 3.3D). These flaps are fixed proximally to the wall of the pharynx and extend with their anterior part to the openings of the respective parts of the gland. From their location, dimension and arrangement these flaps seem to be able to close the pharynx just proximally to the opening of the reservoir (pharynx closing flaps in Fig. 3.3D). Therefore, they might facilitate the transfer of secretion between the gland and the mouth opening. This scenario is hypothetical, however, and the exact assignment and functioning of the flaps remain to be shown.

The SEM investigations confirmed the results of the histological investigations and revealed that the cells of the epithelium form dense foldings into the lumen of the reservoir (Fig. 3.4). The rims of these folds bear numerous thin hairs that extend into the lumen of the reservoir and are variable in length (approx. 10 - 60 µm). The function of these hairs is not yet known. The walls of the pharynx show similar but more regular foldings and the hairs are shorter (not shown).

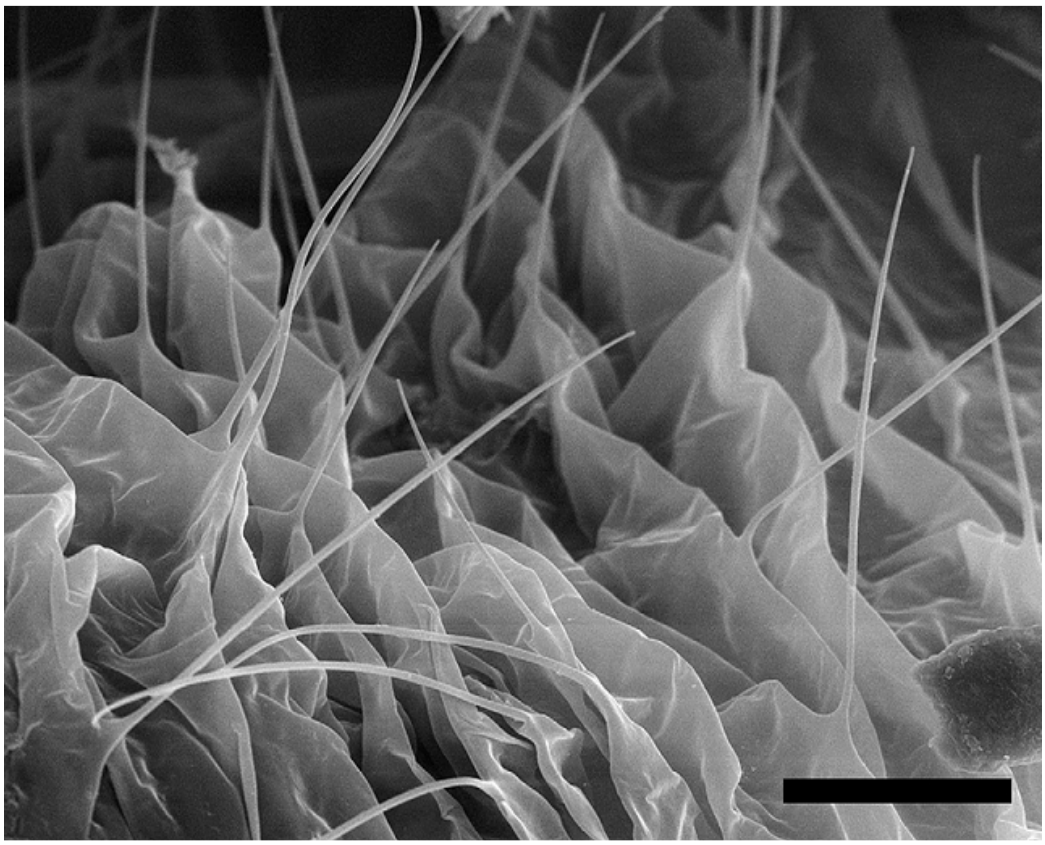

Fig. 3.4: Scanning electron micrograph of the inner surface of the postpharyngeal gland of male *P. triangulum* (scale bar 20 µm). Note the epithelial folds and hairs.

The TEM analysis confirmed the finding that the wall of the gland reservoir is formed by a monolayered epithelium lined by an inner intima that shows the typical ultrastructure of an insect cuticle (Fig. 3.5). Owing to the epithelial foldings and hairlike extensions the epithelial thickness is very variable. The TEM investigation did not reveal any gland ducts that might discharge into the lumen of the reservoir. The cells of the epithelium are connected by septate desmosomes. They contain some rough and some smooth endoplasmatic reticulum. Some cells contain a large number of mitochondria but others have only few mitochondria. Multilamellar bodies and Golgi apparatus could rarely be seen. There were only few vesicles

in the cells and no microvilli on the inner side of the epithelium. There were, however, basal invaginations on the outer side of the epithelium (Fig. 3.5C).

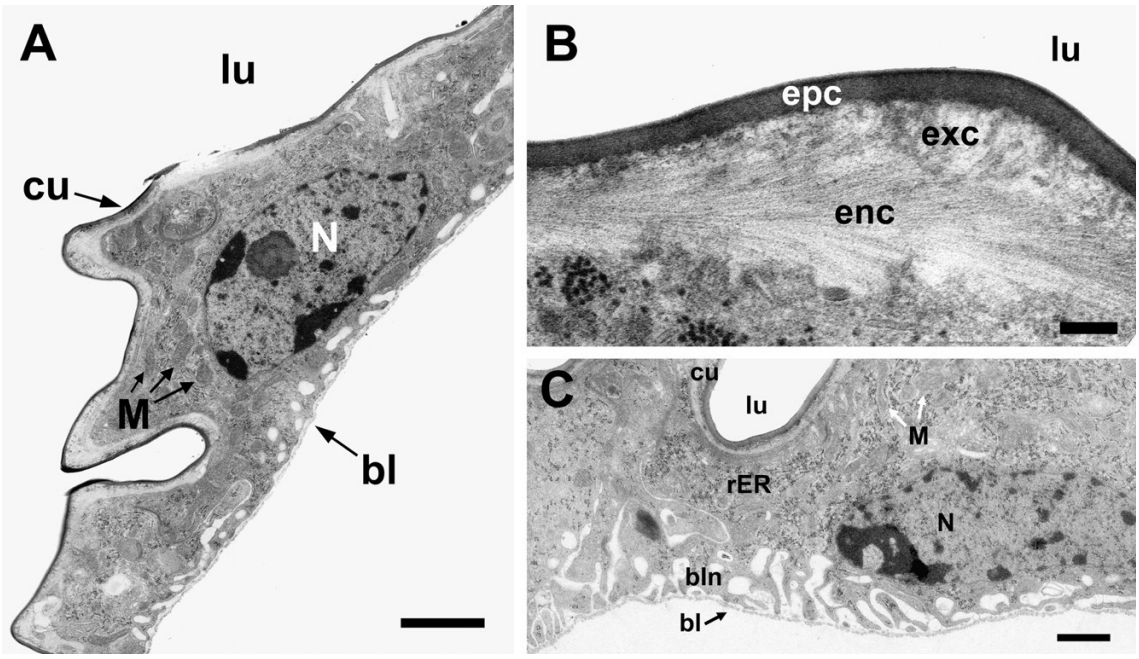

Fig. 3.5: Transmission electron micrographs of the postpharyngeal gland epithelium (A, scale bar 4 mm and C, scale bar 2 µm) and cuticle (B, scale bar 0.2 µm) of a male *P. triangulum*. The basal invaginations of the epithelium suggest an uptake of substances from the hemolymph. bl, basal lamina; bIn, basal invaginations; cu, cuticle; enc, endocuticle; epc, epicuticle; exc, exocuticle; lu, lumen of the PPG; M, mitochondria; N, nucleus; rER, rough endoplasmatic reticulum.

3.3.2 Chemistry

The gland reservoir samples contained 35 previously described (Kroiss et al. 2006; Schmitt et al. 2003) pheromonal substances (Fig. 3.6A). Some of the very minor HCs that had been found as components of the gland content in another study (Kroiss et al. 2006) could not be detected, most probably because we analyzed single males with the consequence that the amounts of some substances were below the detection limits of the analytical set-up.

In the cuticle extracts we characterized 19 peaks (Fig. 3.6B). All these substances also occur in the gland. The concentration of HCs in the cuticle extracts was considerably lower than in the gland extracts and it is likely that some of the HCs that we found in the pheromone but not in the cuticle extracts were below the detection limits of our GC-MS. The amounts of the

methyl alkanes were too low to determine the position of the methyl group. Except for Δ-16-pentacosen-8-one none of the pheromonal substances with functional groups were present in the cuticle extracts.

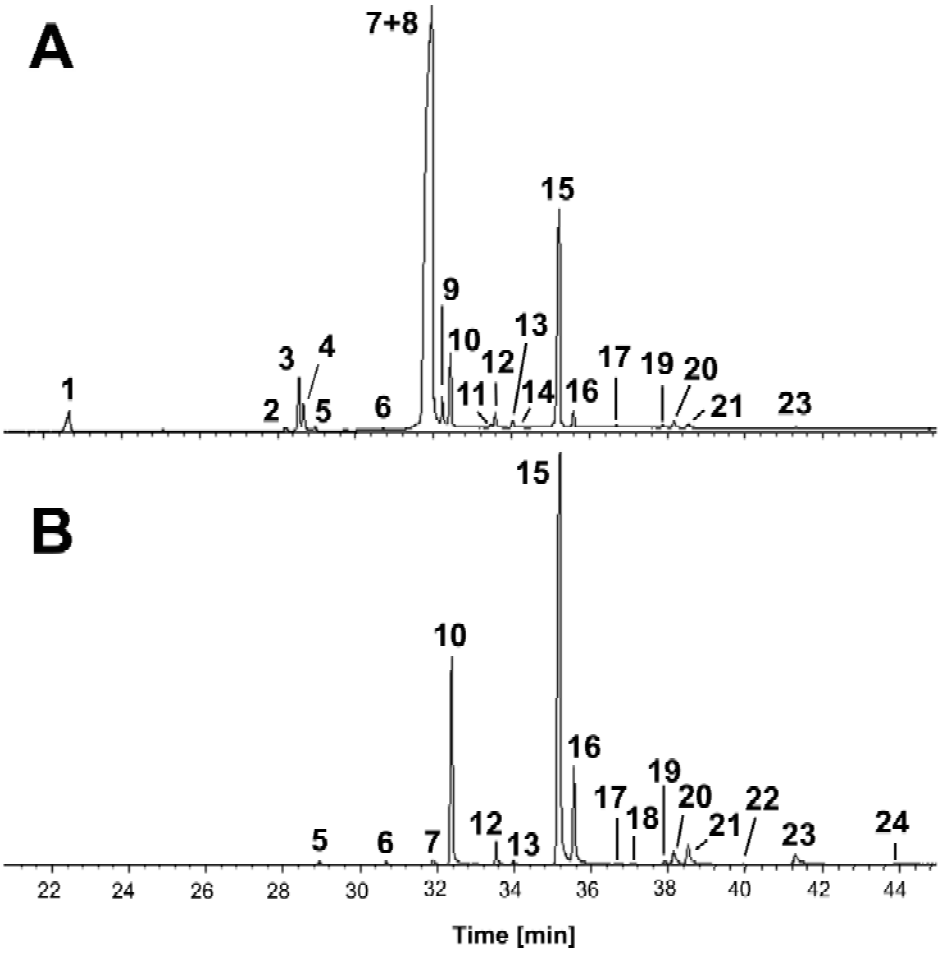

Fig. 3.6: Gas chromatograms of hexane extracts of an individual male *P. triangulum*. (A) PPG content. (B) Epicuticle. The numbers in the chromatograms refer to the following compounds: 1, (*S*)-2,3-dihydrofarnesoic acid; 2, (*Z*)-9-octadecen-1-ol; 3, 10-nonacosen-2-one; 4, 1-octadecanol; 5, heneicosane; 6, docosane; 7, $\Delta^{x,y}$-tricosadiene + (*Z*)-9-tricosene + (*Z*)-7-tricosene; 8, (*Z*)-11-eicosenol; 9, 1-eicosanol; 10, tricosane; 11, unidentified substance 1; 12, $\Delta^{x,y}$ -tetracosadiene + (*Z*)-9-tetracosene + (*Z*)-7-tetracosene; 13, tetracosane; 14, unidentified substance 2; 15, $\Delta^{x,y}$-pentacosadiene + (*Z*)-9-pentacosene + (*Z*)-7-pentacosene; 16, pentacosane; 17, (*Z*)-9-hexacosene; 18, hexacosane; 19, Δ-16-pentacosen-8-one; 20, $\Delta^{x,y}$-heptacosadiene + (*Z*)-9-heptacosene + (*Z*)-7-heptacosene; 21, heptacosane; 22, octacosane; 23, nonacosane; 24, hentriacontane. Please note that owing to their very low quantities the peaks of the substances 13-methyl pentacosane, 11-methyl pentacosane, 7-methyl-pentacosane, hexacosane and octacosane are not visible in one or both of the chromatograms of one individual male.

For the correlation and regression analysis we included all HC peaks that were present both in the gland and on the cuticle and that could unambiguously be identified. In general, the (*Z*)- 9 and (*Z*)-7 alkenes as well as the alkadiene of the same chain length could not be entirely separated in the chromatograms and were thus treated as one peak. The (*Z*)-9-alkene

was always the dominant peak among the three. In the chromatograms of the gland extracts, the peaks of Δx,y-tricosadiene, (*Z*)-9-tricosene, and (*Z*)-7-tricosene were hidden under the huge peak of (*Z*)-11-eicosen-1-ol and could not be separated satisfactorily for a quantitative analysis. They were thus excluded from the analyses. The very minor compounds Δ-16-pentacosen-8-one and octacosane were only present in traces in most of the gland extracts and not detectable at all in most of the cuticle extracts and were thus also excluded from the analyses. The remaining 12 peaks (Aitchison- and log-transformed) were subjected to the correlation and regression analyses.

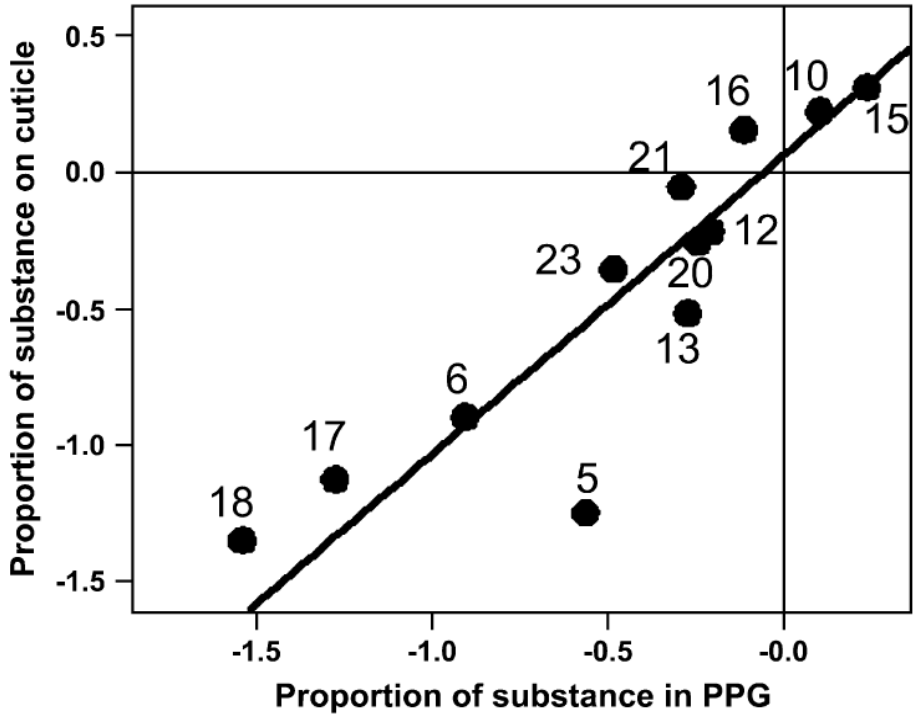

Fig. 3.7: Correlation between the mean relative amount (peak area Aitchison transformed, see text) of a particular hydrocarbon in the PPGs and on the cuticles of beewolf males (r^2 = 0.801, n = 11, p < 0.001). The trend line was generated using reduced major axis regression (y = 1.091x + 0.062). The data are based on extracts of 14 males and the following 12 peaks (the numbers correspond to the numbers given in Fig. 3.6): 5, heneicosane; 6, docosane; 10, tricosane; 12, tetracosene; 13, tetracosane; 15, pentacosenes + pentacosadiene; 16, pentacosane; 17, hexacosene; 18, hexacosane; 20, heptacosenes + heptacosadiene; 21, heptacosane; 23, nonacosane.

The relative amounts of substances in the gland reservoirs showed a strong linear correlation with the corresponding substances on the cuticles (Fig. 3.7). The slope of the RMA regression was 1.091 (95% confidence intervals: 0.748 - 1.433). The intercept of the regression line was 0.062 (95% confidence intervals: -0.175 - 0.299). Thus, there was no significant deviation from direct proportionality.

3.4 DISCUSSION

Our findings strongly support the hypothesis that the large cephalic gland reservoir of male *P. triangulum* is a PPG. Despite the difference in overall appearance, there is considerable similarity between the PPG of male European beewolves and their female conspecifics as well as ants. As in female *P. triangulum* and ants, the presumptive PPG of male beewolves originates from the posterior part of the pharynx and takes up a considerable fraction of the head-capsule volume. Whereas in ants the PPG is made up of only one paired structure (Peregrine et al. 1973; Soroker et al. 1995), in both sexes of *P. triangulum* the reservoir comprises two parts (the upper and the lower part; Strohm et al. 2007 this study). The overall appearance of these two parts of the reservoir is sexually dimorphic, however. Whereas in males both the upper and the lower part of the reservoir consist of two symmetrical halves, in females only the upper part shows this subdivision, and the lower part consists of a single small sac-like structure (Strohm et al. 2007). Furthermore, in contrast to the overall glove-like structure of the upper part of the PPG in female *P. triangulum* (Strohm et al. 2007) and ants (Lucas et al. 2004; Peregrine et al. 1973; Soroker et al. 1995), the upper part of the PPG of male *P. triangulum* consists of unbranched simple straight tubes.

The epithelium that forms the wall of the gland reservoir is monolayered in male and female *P. triangulum* (Strohm et al. 2007) and in ants (Peregrine et al. 1973; Soroker et al. 1995). In both sexes of the beewolf, the epithelium carries long hairs that extend into the lumen of the reservoir and the inner surface is lined with cuticle. With the exception of the hairs, which are not present in ants, these results support the hypothesis that the large cephalic reservoir of male European beewolves is a PPG.

The PPG of ants typically contains long-chain straight and methyl-branched HCs that are sequestered from the hemolymph and/or taken up from the cuticle during self-grooming (Hefetz et al. 2001; Lucas et al. 2004; Soroker et al. 1998; Soroker & Hefetz 2000; Soroker et al. 1995; Soroker et al. 1994). In female *P. triangulum* the PPG content also comprises long-chain straight and methyl-branched HCs (plus additionally long-chain unsaturated ketones) and there are indications that these are likewise sequestered from the hemolymph (E. Strohm, G. Herzner, T. Schmitt, unpublished). The glove-like gland shape may facilitate the efficient sequestration of HCs from the hemolymph by enlarging the surface of the gland reservoir. Both in ants (Bagneres & Morgan 1991; Do Nascimento et al. 1993; Lucas et al. 2004; Soroker et al. 1995; Soroker et al. 1995) and female beewolves (E. Strohm, G. Herzner, T. Schmitt, unpublished) the HCs in the PPG match those extractable from the cuticle. In

male *P. triangulum* the PPG functions as reservoir of the marking pheromone (Kroiss et al. 2006). As in ants and females it contains (among others) several straight and methyl-branched long-chain HCs. In a previous study on the chemistry of the marking pheromone based mainly on head extracts (Schmitt et al. 2003) we did not include most of the minor HCs. During this previous study we were not aware of the existence of the PPG in male beewolves and we thought that the minor HCs in our head extracts came from the cuticle rather than the cephalic glands. Here and in Kroiss et al. (2006) we show that they are indeed present in the PPG and we thus include them in the description of the marking pheromone. We also found a high congruence between the HCs from the reservoir and the cuticle. This provides further evidence for a homology of the male reservoir with the PPG of ants and female beewolves.

Although in ants the cuticular HC composition generally appears to be congruent with that of the PPG, some deviations from this rule have also been documented (Lucas et al. 2004; Soroker & Hefetz 2000). However, these differences between PPG and cuticular HCs are mostly quantitative in nature. In male *P. triangulum* we found a high congruency concerning the HC fraction of the PPG and cuticular HCs. In addition to the HCs, the PPG contained several compounds with functional groups that did not occur on the cuticle, i.e. there are large qualitative differences between the gland and the cuticle. This raises the question where this blend of compounds in the PPG of male beewolves comes from.

Chemical analyses of the cuticle as well as preliminary analyses of the hemolymph (M. Kaltenpoth, unpublished data) suggest that the HCs might be either sequestered from the cuticle or from the hemolymph or both. Owing to the large size of the male PPG and the low proportion of HCs in its content (Kroiss et al. 2006 this study) the surface of the gland might be sufficiently large to allow for the sequestration of the HCs, even if the reservoir lacks the surface enlargement of the glove-like female PPG. Sequestration of HCs from the hemolymph by exocrine glands has also been proposed for other species (Dufour's gland of honey bee queens: Gozansky et al. 1997; pheromone glands of moths: Jurenka et al. 2003; Schal et al. 1998). The pheromonal compounds with functional groups do not occur in the hemolymph of male *P. triangulum* (M. Kaltenpoth, unpublished data) and must thus come from somewhere else. The lack of typical class 3 gland cells and the thin epithelium with no signs of high glandular activity suggest that these compounds are not synthesized by the PPG itself. Instead, we hypothesize that they are produced in the large mandibular glands (Fig. 3.2, E. Strohm and W. Goettler, unpublished) and transferred to the PPG via the pharynx. Since the substances with functional groups do not occur on the cuticle, it is rather unlikely that male beewolves apply the PPG secretion to their cuticle via self-grooming as demonstrated, e.g. for the ant *Cataglyphis niger* (Soroker & Hefetz 2000).We cannot preclude, however, that the

HCs take the opposite route, i.e. that they are incorporated into the PPG during self-grooming, a mechanism proposed, e.g. for the ants *Camponotus vagus* (Meskali et al. 1995) as well as *Pachycondyla apicalis* and *P. villosa* (Hefetz et al. 2001; Lucas et al. 2004). The marking pheromone that is stored in the PPG thus seems to be a mixture of substances with different origins.

The finding of a pheromone storing PPG in males of a solitary wasp is surprising. The 3D reconstruction visualizes the impressive size of the pheromone producing and storing glands in male European beewolves. Together, the PPG and the mandibular gland make up approximately one-third of the head-capsule volume. The size and content of the male PPG are most probably shaped by strong sexual selection. Whereas the composition of the pheromone might have been influenced by receiver bias processes (Endler & Basolo 1998; Herzner et al. 2005), the extraordinary size of the glands and consequently the exceptional large amounts of pheromone suggest the involvement of runaway processes (Fisher 1930) in the exaggeration of this secondary sexual character (see also Herzner 2004).

It is an intriguing yet common phenomenon that glands that serve particular functions in solitary species (like the PPG in male and female beewolves) have been modified to serve 'social functions' in social species (like the PPG in ants). The Dufour's gland, for example, serves numerous different functions in various solitary and social species. In solitary bees of the families Colletidae, Andrenidae, and Anthophoridae, females use the Dufour's gland secretion to line the walls of underground brood cells with hydrophobic substances to maintain favorable microclimatic conditions for their progeny (e.g. Vander Wall 1990). In ants, the Dufour's gland contains (among others) trail, recruitment, and queen pheromones (e.g. Bestmann et al. 1995; Blatrix et al. 2002; Edwards & Chambers 1984). In honeybee queens, it contains substances that elicit retinue behavior by workers (Katsav-Gozansky et al. 2001). A further example is the poison gland that first served to paralyze hosts or prey (Quicke 1997), then evolved an additional defensive function in brood caring solitary species (Wilson 1972) and has finally gained a function in recruitment (Kohl et al. 2001) and trail establishment (Morgan et al. 1992) in ants.

The occurrence of PPGs in beewolves is remarkable, since it has long been assumed that PPGs are restricted to the family Formicidae (Hölldobler & Wilson 1990; Lenoir et al. 1999; Schoeters & Billen 1997; Soroker et al. 1995), which is phylogenetically not closely related to crabronid wasps (Brothers 1999). In ants the PPG harbors a heritable blend of cuticular HCs that allows for nestmate recognition and thus contributes to colony integrity, a problem idiosyncratic to social species. Lenoir and coworkers (1999) thus speculated about the evolutionary origin of the PPG: 'Is there a cuticular lipid storing gland in solitary species, or

has it evolved specifically in ants' to allow the formation of a colony signature and thus nestmate recognition? Here and in a further study (Strohm et al. 2007) we demonstrate the existence of such a lipid storing gland in a solitary digger wasp. Based on current knowledge it remains unclear whether the PPGs of beewolves and ants are derived from a single evolutionary root or evolved by independent convergent evolution (for a detailed discussion of this issue see Strohm et al. 2007).

In any case, another interesting question that arises is whether the chemical composition of the PPG content in the European beewolf has a genetic basis and thus the potential to enable kin recognition. In P. *triangulum* the PPG is involved in brood care by females (Strohm & Linsenmair 2001) and in mate attraction by males (Kroiss et al. 2006; Schmitt et al. 2003). It is as yet unknown whether the composition of the PPG secretion of females is heritable. In male *P. triangulum*, however, the overall composition of the PPG content, i.e. the sex pheromone, in fact varies with kinship (Herzner et al. 2006). Taking into account that the ability to recognize nestmates (which usually involves kin recognition to some degree) by virtue of chemical cues is one of the key characteristics of social insects, a genetically based composition of the PPG content of a solitary species, such as *P. triangulum*, could represent a crucial preadaptation for the evolution of nepotism and sociality.

In conclusion, our findings strongly support the existence of a PPG, a gland that was thought to be idiosyncratic to ants, where it guarantees the integrity of the social group, in males of a solitary wasp, the 'lone beewolf' *P. triangulum*.

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CHAPTER 4

THE ODOR OF ORIGIN: KINSHIP AND GEOGRAPHICAL DISTANCE ARE REFLECTED IN THE MARKING PHEROMONE OF MALE BEEWOLVES (*PHILANTHUS TRIANGULUM* F., HYMENOPTERA, CRABRONIDAE).

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4.1 SUMMARY

Background: Pheromones play an important role for mate finding and courtship in many insects. In species where males are the signaling sex, females are expected to choose among potential mates with regard to the emitter's quality and/or genetic compatibility. One important aspect is the balance between negative and positive effects of in- vs. outbreeding. In the present study, we aimed to assess the potential of the territory marking pheromone of European beewolves as an indicator for genetic compatibility in the context of female choice.

Results: We analyzed the sex pheromone composition of male European beewolves (*Philanthus triangulum* F., Hymenoptera, Crabronidae) from eight different locations across Central Europe (six in Germany, one in England, and one in Italy). The pheromone constitutes a complex blend of various long-chain hydrocarbons (alkanes, alkenes, alcohols, ketones, and a carbon acid). We demonstrate that pheromone composition differs significantly among distant populations (regional scale), among subpopulations (local scale) and between families within subpopulations. The differences in the pheromone blend are positively correlated with geographical distances as might be expected according to an isolation-by-distance model. On a local scale, family membership has a larger effect on pheromone composition than subpopulation affiliation, while the reverse is true for the regional scale.

Conclusion: Our results show that male pheromones can contain information on both kinship and geographical origin that may be used by females to choose adaptively among potential mates on the basis of their genetic distance.

4.2 BACKGROUND

In many animals, sexual signals vary with the degree of kinship as well as with geographical distribution. This has been shown for numerous species with acoustical courtship signals (Nelson & Soha 2004; Packert & Martens 2004; Westcott & Kroon 2002), but also for several taxa with sex pheromones (Gemeno et al. 2000; Grosman et al. 1997; Huang et al. 1998; McElfresh & Millar 2001; Yatsynin et al. 1996). Previous studies on chemical signals, however, have focused on pheromones produced by females; evidence for geographical variation in male sex pheromones is largely lacking (but see Aldrich et al. 1989; but see Hamilton et al. 2005; Ryan et al. 1995; Watts et al. 2005).

Since there is usually a conflict of interest between the sexes (Trivers 1972), male sex pheromones are expected to underlie completely different selective pressures than female pheromones (Phelan 1992; Phelan 1997). Male sexual signals often enable females to choose adaptively among potential mates by providing information on species affiliation and mate quality (Droney & Hock 1998; Jones & Hamilton 1998; O'Loghlen & Rothstein 2003; Slater 2003). If males vary in their ability to provide essential resources to the females (Halliday 1983; Vahed 1998) or in their parasite or disease load (Clayton 1991; Hamilton & Zuk 1982; Penn & Potts 1998) and such differences in quality are indicated in the males' signals females could benefit directly by choosing a high-quality male. Females may also benefit indirectly, if offspring quality depends on the genetic background of the male. Several models have been proposed to explain female choice based on indirect benefits, the most prominent of these being the “good genes” model (Andersson 1994; Møller & Alatalo 1999; Wilkinson et al. 1998), and the model of the “best compatibility” (Colegrave et al. 2002; Halliday 1983; Tregenza & Wedell 2000).

The genetic compatibility of a mate depends, among other things, on the degree of kinship which ranges from strict inbreeding to extensive outbreeding, both of which have certain advantages (Partridge 1983) and disadvantages (Bateson 1983; Pusey & Wolf 1996). According to the model of optimal outbreeding, females should choose a mate of a certain

genetic distance to balance negative effects of inbreeding and outbreeding (Alexander 1977; Bateson 1983; Bischof 1972).

Male European beewolves establish small territories in the vicinity of female nest aggregations, and territories do not contain any resources essential for beewolf females (Simon-Thomas & Poorter 1972; Strohm 1995). The males apply a marking pheromone from a cephalic gland (the postpharyngeal gland, PPG) onto plants within their territory and defend the territory against intruding males in combat flights without physical contact (Evans & O'Neill 1988; Kroiss et al. 2006; Schmitt et al. 2003; Simon-Thomas & Poorter 1972; Strohm 1995; Strohm & Lechner 2000). The marking pheromone of male European beewolves comprises a complex blend of up to 55 compounds (Kroiss et al. 2006; Schmitt et al. 2003) that might contain important cues for females to assess male quality and/or compatibility.

Behavioral observations provide clear evidence that the marking pheromone of beewolf males attracts receptive females to the males' territories (Evans & O'Neill 1988). Females approach territories in a zigzagging flight pattern from the downwind side, probably orienting towards the windborne pheromone (Evans & O'Neill 1988). Copulations usually occur within the males' territories (Evans & O'Neill 1988; Simon-Thomas & Poorter 1972; Strohm 1995) and seem to be under the control of females since they can easily repel unwanted males by virtue of their larger body size (Evans & O'Neill 1988) or refuse copulation by bending their abdomen tip downwards (E. Strohm, pers. observation). Territories of different males are often aggregated, thereby constituting a lek situation in which the females have an ideal opportunity to compare among potential mates and choose the most suitable (Evans & O'Neill 1988; Simon-Thomas & Poorter 1972). Since the copulation is not preceded by any kind of visual display, female choice appears to be, at least predominantly, based on information obtained from the male sex pheromone (E. Strohm, M. Kaltenpoth, J. Kroiss unpublished data). The amount and composition of the male PPG content have been shown to differ between families (Herzner et al. 2006) and to vary with the age of the males (Kaltenpoth & Strohm 2006).

Beewolves have good flying abilities and it is likely that individuals from different (sub)populations meet in the field. Thus, the discrimination between males belonging to different (sub)populations may be an important factor influencing female mate choice decisions. Using combined gas chromatography-mass spectrometry (GC-MS), we investigated whether the marking pheromone of male European beewolves varies between populations, between subpopulations, and among families within subpopulations in a way that might provide a basis for female choice. We compared the relative effects of population

and family association on the composition of the pheromone and discuss the consequences for optimal mate choice.

4.3 RESULTS

4.3.1 Pheromone amount and composition

Using coupled GC-MS, we found a total of 25 substances in the male sex pheromone: (*S*)-2,3-dihydrofarnesoic acid (DHFS hereafter); (*Z*)-9-octadecen-1-ol; (*Z*)-10-nonadecen-2-one; 1-octadecanol; heneicosane; "Unidentified substance 1" (unknown 1); "Unidentified substance 2"; docosane; (*Z*)-11-eicosen-1-ol; a mixture of (*Z*)-9-, (*Z*)-7-tricosene, and Δx,y-tricosadiene; 1-eicosanol; tricosane; a mixture of (*Z*)-9-, (*Z*)-7-tetracosene, and Δx,y-tetracosadiene (C24en); tetracosane (C24an); a mixture of (*Z*)-9-, (*Z*)-7-pentacosene, and Δx,y-pentacosadiene (C25en); pentacosane (C25an); a mixture of 7-, 11-, and 13-methyl pentacosane (m-C25an); (*Z*)-9-hexacosene (C26en); hexacosane (C26an); Δ-16-pentacosen-8-one (C25one); a mixture of (*Z*)-9-, (*Z*)-7-heptacosene, and Δx,y-heptacosadiene (C27en); heptacosane (C27an); octacosane (C28an); nonacosane (C29an); hentriacontane (C31an). Several peaks had to be combined for the analysis, because they were not always clearly separated by the GC-MS. This applies to (*Z*)-9-octadecen-1-ol and (Z)-10-nonadecen-2-one (C19enone hereafter), 1-octadecanol and heneicosane (C18anol), "Unidentified substance 2" and docosane (C22ane), (*Z*)-11-eicosen-1-ol and tricosenes/tricosadiene (C20enol), and 1-eicosanol and tricosane (C23ane). Thus, the 25 detected substances were reduced to 20 variables that were included in the analysis. This procedure is conservative with regard to the hypotheses tested. The total amount of pheromone extracted from *P. triangulum* males varied between 101 and 2508µg (mean ± SD = 655 ± 377µg). In both data sets, the sampled populations differed significantly in their total amount of pheromone (data set 1: ANOVA, $F_{5, 254} = 6.66$, $p < 0.01$; data set 2: ANOVA, $F_{4, 128} = 3.99$, $p < 0.01$).

4.3.2 Chemical dimorphism

The pheromone composition showed a distinct dimorphism (see Kroiss et al. 2006 for a detailed description of the dimorphism). The two morphs differ mainly in the relative proportions of pentacosene (mixture of isomers with (*Z*)-9-pentacosene as the main component) and heptacosene (mixture of isomers with (*Z*)-9-heptacosene as the main component), and they can be distinguished unambiguously by the relative amount of heptacosene, which shows a clearly bimodal distribution (Kroiss et al. 2006). The morph

with the high proportion of pentacosene (in the following called C_{25}-type) was overall the more common type (79.1% of all males) compared to the one having approximately equal proportions of penta- and heptacosene (C_{25}/C_{27}-type in the following; 20.9% of all males) (see also Kroiss et al. 2006). The frequency of the C_{25}-type varied considerably between the sampled populations from 8.3 to 100.0% in the sampled populations (mean ± SD = 72.6 ± 26.3%). Since chance variations in the proportion of C_{25}- and C_{25}/C_{27}-type males among families and among populations can greatly influence the outcome of statistical analyses on the chemical differentiation, all of the following analyses were performed on C_{25}- and C_{25}/C_{27}-type individuals combined as well as on C_{25}-type individuals only.

4.3.3 Population differentiation

The analysis of geographical variation in the male beewolf sex pheromone was conducted with two data sets on two geographical scales. The first data set (data set 1) was focused on a local scale (subpopulation level), the second data set (data set 2) with emphasis on a regional scale (population level). Populations in both data sets and, thus, on two different spatial scales could be significantly separated by discriminant analyses (DAs) (Table 4.1, Fig. 4.1 and 4.2). This was irrespective of the inclusion or omission of the C_{25}/C_{27}-type in the analysis. Classification of DA revealed that 45.0 to 56.1% of males were correctly assigned to the populations, depending on the data set and inclusion or omission of the C_{25}/C_{27}-type (20.0 or 25.0% correct classifications would have been expected by chance). Despite the higher number of groups in the DA, the classification results were generally more accurate for the samples on the regional than on the local scale, indicating that the chemical distances were positively correlated with the geographical scale.

Table 4.1: Population and family differentiation by principal components and discriminant analyses. "Type" indicates, whether only the C_{25}-type or both C_{25}- and C_{25}/C_{27}-type were included in the analysis. For PCAs, the number of factors as well as the cumulative explained variance is given. For DAs, the number of functions, Wilk's-λ, χ^2, degrees of freedom, p-value, and the percentage of correct classifications by DA are given. (Population abbreviations: W: Würzburg, Germany; WB: Würzburg, Biocenter, Germany; WC: Würzburg, City, Germany; V: Veitshöchheim, Germany; R: Retzbach, Germany; D: Düsseldorf, Germany; I: Vizzola Ticino, Italy; E: Puttenham, UK).

						PCA			Discriminant analysis				
Scale	(Sub)Population(s)	Data set	Families	Type	n	Fact.	Expl.Var.(%)	Funct.	Wilk's-λ	X^2	df	p	Corr.class.(%)
Regional	W, S, D, I, E	2	-	both	133	7	84.07	4	0.531	79.65	28	**0.000**	52.6
Regional	W, S, D, I, E	2	-	C_{25}	107	7	83.79	4	0.485	72.40	28	**0.000**	56.1
Local	W_B, W_C, V, R	1	-	both	191	6	80.58	3	0.604	93.30	18	**0.000**	45.0
Local	W_B, W_C, V, R	1	-	C_{25}	175	6	80.41	3	0.611	83.14	18	**0.000**	49.7
Family	W_B	1	4	both	45	7	84.87	3	0.292	47.43	21	**0.001**	60.0
Family	W_B	1	4	C_{25}	44	7	86.09	3	0.240	53.44	21	**0.000**	75.0
Family	W_C	1	7	C_{25}	74	5	79.76	5	0.097	156.25	30	**0.000**	58.1
Family	W	2	4	C_{25}	36	6	84.62	3	0.206	47.44	18	**0.000**	66.7
Family	V	1	3	both	28	4	81.03	2	0.557	13.73	8	0.089	60.7
Family	V	1	3	C_{25}	24	4	77.46	2	0.614	9.51	8	0.301	62.5
Family	S	1	3	both	28	4	81.07	2	0.116	50.62	8	**0.000**	89.3
Family	S	2	3	both	20	3	66.36	2	0.358	16.45	6	**0.012**	60.0

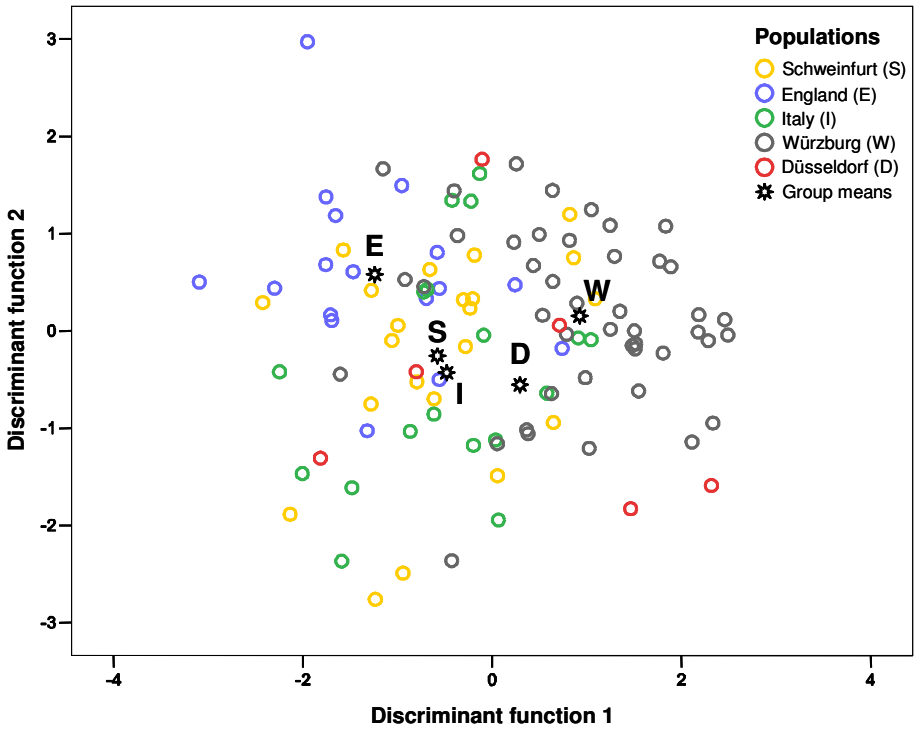

Fig. 4.1: Discriminant analysis of geographical variation of the sex-pheromone on the regional scale (data set 2, five populations, C_{25}-type only). Despite some overlap, the populations are significantly separated (see Table 4.1 and text for details).

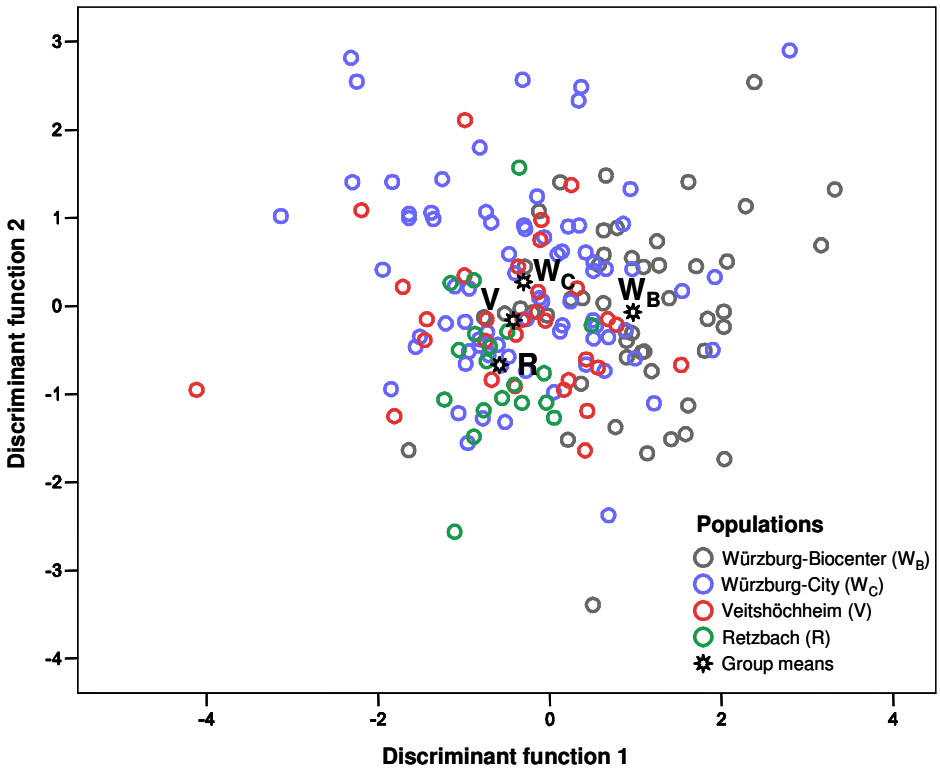

Fig.4.2: Discriminant analysis of geographical variation of the sex-pheromone on the local scale (data set 1, four subpopulations, C_{25}-type only). Despite broad overlap, the populations are significantly separated (see Table 4.1 and text for details).

4.3.4 Family differentiation

Families within populations could be significantly separated in six out of eight populations by DAs (Table 4.1, Fig. 4.3). Individual males were correctly classified in 58.1 to 100.0% of cases (14.3 to 50.0% correct classifications would have been expected by chance). Although DAs were not always significant, the overall classifications of families within populations were more accurate than classifications between populations even if different numbers of groups in the DA are taken into account. Thus, males belonging to different populations and males belonging to different families within a population can be separated from each other on the basis of quantitative differences in some of the pheromone compounds.

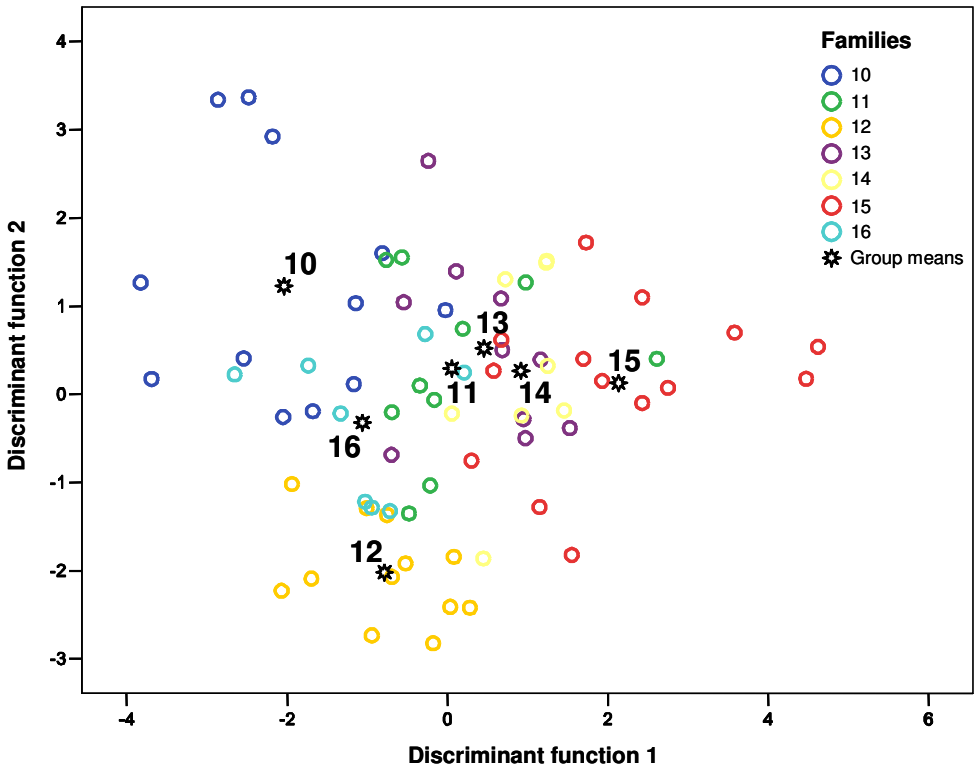

Fig. 4.3: Discriminant analysis of the variation of the sex-pheromone on the family level (data set 1, one population: Würzburg City, C_{25}-type only). Despite some overlap, the families are significantly separated (see Table 4.1 and text for details).

4.3.5 Relative effects of family and population affiliation on pheromone composition

To assess the relative effects of family and population affiliation on the pheromone composition of male European beewolves, we conducted a multivariate nested ANOVA on the

Aitchison-transformed relative peak areas with family membership as a nested factor within populations. Both family and population affiliation had significant effects on the pheromone composition in each dataset, regardless of the omission or inclusion of C_{25}/C_{27}-type males in the analysis (Table 4.2). Together, family and population affiliation explained between 11.4 and 90.8% of the variance in peak areas. The distribution of η^2-values did not deviate significantly from a normal distribution for any of the analyses (Kolmogorov-Smirnov tests: $Z \leq 1.16$, $p \geq 0.135$ for all tests).

On a local scale (data set 1), family membership explained a significantly higher proportion of the variance in pheromone composition than subpopulation affiliation (paired t-tests, C_{25}- and C_{25}/C_{27}-type: $t_{19} = -6.22$, $p < 0.001$; C_{25}-type only: $t_{19} = -6.17$, $p < 0.001$). On a regional scale (data set 2), however, this effect was reversed, with population affiliation explaining more of the variance, although this effect was only significant when both C_{25}- and C_{25}/C_{27}-type males were included (paired t-tests, C_{25}- and C_{25}/C_{27}-type: $t_{19} = 2.11$, $p = 0.048$; C_{25}-type only: $t_{19} = 1.84$, $p = 0.081$).

Table 4.2: Relative effects of family and (sub)population affiliation on pheromone composition in male European beewolves. Proportions of variance explained by family and population membership in nested MANOVAs are estimated for each peak by partial η^2-values. MANOVA results are given for each data set, including and excluding C_{25}/C_{27}-type males, respectively (see text for peak abbreviations).

Data included in MANOVA	**Data set 1: Local C_{25}- and C_{25}/C_{27}-type**		**Data set 1: Local C_{25}-type only**		**Data set 2: Regional C_{25}- and C_{25}/C_{27}-type**		**Data set 2: Regional C_{25}-type only**	
Peak	**η^2(Pop)**	**η^2(Fam)**	**η^2(Pop)**	**η^2(Fam)**	**η^2(Pop)**	**η^2(Fam)**	**η^2(Pop)**	**η^2(Fam)**
DHFS	0.024	0.110	0.010	0.109	0.041	0.118	0.084	0.101
C19enone	0.089	0.243	0.086	0.250	0.439	0.060	0.462	0.105
C18anol	0.118	0.166	0.133	0.168	0.268	0.162	0.188	0.191
unknown1	0.055	0.074	0.033	0.081	0.273	0.070	0.207	0.076
C22ane	0.024	0.302	0.025	0.337	0.053	0.200	0.040	0.263
C20enol	0.088	0.063	0.083	0.072	0.137	0.085	0.152	0.079
C23ane	0.036	0.079	0.056	0.083	0.336	0.190	0.308	0.244
C24ene	0.044	0.354	0.012	0.376	0.069	0.185	0.162	0.139
C24ane	0.085	0.200	0.060	0.200	0.116	0.033	0.083	0.036
C25ene	0.001	0.190	0.016	0.217	0.062	0.156	0.126	0.154
C25ane	0.062	0.165	0.026	0.136	0.161	0.071	0.081	0.079
mC25ene	0.152	0.327	0.119	0.330	0.174	0.094	0.232	0.098
C26ene	0.001	0.182	0.107	0.301	0.284	0.072	0.307	0.096
C26ane	0.225	0.247	0.209	0.230	0.146	0.056	0.093	0.045
C25enone	0.012	0.239	0.012	0.243	0.222	0.289	0.397	0.511
C27ene	0.014	0.188	0.105	0.276	0.255	0.074	0.178	0.037
C27ane	0.205	0.388	0.191	0.397	0.133	0.141	0.091	0.083
C28ane	0.074	0.141	0.066	0.119	0.046	0.085	0.051	0.080
C29ane	0.080	0.166	0.045	0.165	0.219	0.140	0.168	0.080
C31ane	0.076	0.135	0.068	0.135	0.270	0.197	0.285	0.216
MANOVA results:								
Pillai's trace	**0.944**	**3.582**	**0.988**	**3.703**	**2.029**	**2.414**	**2.301**	**2.618**
F	**5.137**	**2.994**	**5.366**	**3.019**	**3.501**	**1.869**	**3.184**	**1.897**
p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**

4.3.6. Correlation between geographical and chemical distance

To test for a correlation between the matrices of geographical and chemical distances of the populations we performed a Mantel test. We detected a strong correlation between geographical and chemical distance for data set 1 and the normalized combination of both datasets irrespective of which combination of populations was used as a reference for normalization and independent of the inclusion or omission of the C_{25}/C_{27}-type in the analysis (Tables 4.3 and 4.4, Fig. 4.4). A Mantel test restricted to data set 2 revealed no significant correlation.

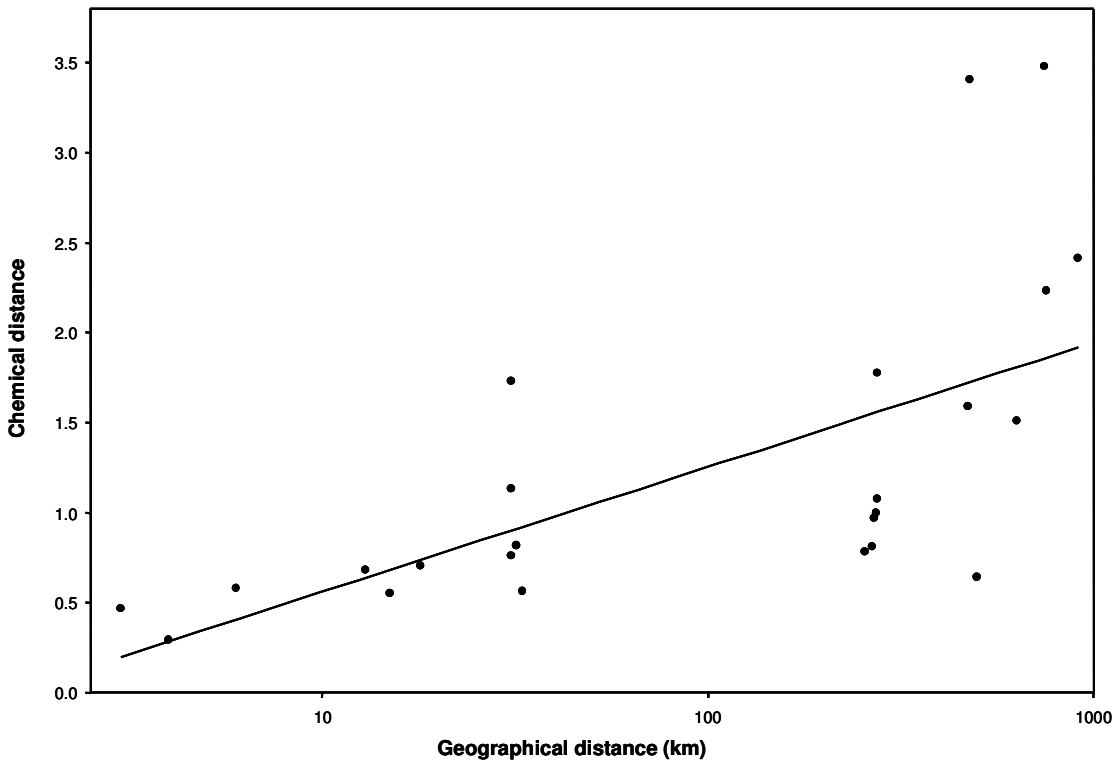

Fig. 4.4: Correlation between pairwise geographic and chemical distances of populations of *P. triangulum* males (data sets 1 and 2; C_{25}-type only; normalization: Würzburg - Düsseldorf; Mantel-test: r = 0.630, p = 0.013; see also Table 4.4 and text for details). The trend line was obtained by linear regression in order to visualize the association.

Table 4.3: Correlation between geographic and chemical distances of populations of *P. triangulum*. Given are regression coefficients (r) and p-values of Mantel tests. The column "type" indicates, whether only the C_{25}-type or both C_{25}- and C_{25}/C_{27}-type were included in the analysis. Data sets 1 and 2 were normalized to Würzburg – Schweinfurt (W–S), Würzburg – Düsseldorf (W–D), or Schweinfurt – Düsseldorf (S–D). For population abbreviations see Table 4.1. P-values < 0.05 are given in bold.

Data set	Scale	Populations	Type	Normalization	Mantel test r^2	Mantel test p
1	Local	W_B, W_C, V, R, S, D	both	-	**0,854**	**0,003**
1	Local	W_B, W_C, V, R, S, D	C25	-	**0,584**	**0,004**
2	Regional	W, S, D, I, E	both	-	0,092	0,254
2	Regional	W, S, D, I, E	C25	-	0,074	0,319
1 and 2	Local+Regional	W, W_B, W_C, V, R, S, D, I, E	both	S-W	**0,468**	**0,008**
1 and 2	Local+Regional	W, W_B, W_C, V, R, S, D, I, E	both	D-W	**0,608**	**0,003**
1 and 2	Local+Regional	W, W_B, W_C, V, R, S, D, I, E	both	S-D	**0,581**	**0,004**
1 and 2	Local+Regional	W, W_B, W_C, V, R, S, D, I, E	C25	S-W	**0,253**	**0,016**
1 and 2	Local+Regional	W, W_B, W_C, V, R, S, D, I, E	C25	D-W	**0,397**	**0,013**
1 and 2	Local+Regional	W, W_B, W_C, V, R, S, D, I, E	C25	S-D	**0,362**	**0,005**

Table 4.4: Geographical and chemical distances between the nine sampled (sub)populations of *P. triangulum*. Chemical distances (normalized to Würzburg – Düsseldorf) are displayed *in italics* in the lower left half of the table, geographical distances [km] in the upper right half of the table. For population abbreviations see Table 4.1.

	W	W_B	W_C	V	R	S	D	I	E
W	-	NA	4	6	18	31	274	472	746
W_B	*NA*	-	4	6	18	31	274	472	746
W_C	*NA*	*0.29*	-	3	15	32	270	473	743
V	*NA*	*0.58*	*0.47*	-	13	33	267	474	740
R	*NA*	*0.70*	*0.55*	*0.68*	-	31	256	485	732
S	*1.73*	*0.76*	*0.82*	*0.57*	*1.13*	-	276	501	758
D	*1.00*	*1.00*	*0.97*	*0.81*	*0.79*	*1.08*	-	635	479
I	*1.59*	*NA*	*NA*	*NA*	*NA*	*0.64*	*1.51*	-	911
E	*3.48*	*NA*	*NA*	*NA*	*NA*	*2.23*	*3.40*	*2.41*	-

4.4 DISCUSSION

In the present study, we investigated inter-individual differences in the pheromone composition of male European beewolves on three different levels: between families, among subpopulations on a local scale, and among geographically distant populations on a regional scale. Our results show that there are significant differences in pheromone composition on all three levels and that the chemical distance between populations is correlated with the geographical distance.

Although local subpopulations as well as geographically distant populations of beewolves could be separated on the basis of the male sex pheromone, only 45 - 56% of the individual males were classified correctly by the discriminant analyses, and chemical profiles of different populations overlapped considerably. However, the existing differences might be sufficient for females to reduce the incidence of outbreeding, especially because the sensitivity of female chemoreceptors and the discriminatory ability of their central nervous system may exceed that of our analytical methods by several orders of magnitude (Angioy et al. 2003; Kaissling 1971). Furthermore, our data provide only a lower boundary for the actual effect of geographical origin on the pheromone composition, since all the animals were reared under identical conditions in the laboratory. In the field, developmental conditions certainly vary between (sub)populations. Differences in environmental factors during larval development have been shown to affect the pheromone composition of male beewolves (K. Roeser-Mueller, M. Kaltenpoth & E. Strohm, unpubl. data). Therefore, actual differences between populations in the field may be much larger than those observed under controlled conditions in the laboratory and might allow females a better discrimination of males from different (sub)populations.

Since beewolves have good flying abilities and are pioneer species that frequently colonize new habitats (Evans 1974), females are likely to encounter males from other local subpopulations in the field (whereas it is unlikely that they encounter individuals from distant populations). Thus, female beewolves may use the information contained in the male pheromone to avoid outbreeding depression. Deleterious effects of extensive outbreeding have been demonstrated in many recent studies (Aspi 2000; Edmands 1999; Peer & Taborskyi 2005), and several hypotheses have been proposed to explain why outbreeding depression occurs (e.g. break-up of coadapted gene complexes, disruption of epistatic interactions, loss of local adaptations, dispersal hazards, and risk of parasite infection; see Bateson 1983; Pusey & Wolf 1996).

Within populations, beewolf male pheromones differ significantly among families (this study and Herzner et al. 2006). The family-related differences may enable females to reduce the chances of mating with close kin and thereby avoid inbreeding depression, which is likely to impose especially high costs on beewolves. Kin recognition in animals is generally mediated by one of three mechanisms: phenotype matching, recognition of genetically compatible mates, or imprinting or learning of the individuals that occur in the same nest or birth place (Fletcher & Michener 1987; Hepper 1986; Pusey & Wolf 1996). The mechanisms by which female beewolves could distinguish between kin and non-kin are unclear (for discussion see Herzner et al. 2006). The discrimination of males from different populations may be possible for females by sampling pheromones from different males in a lek and avoiding individuals

with a pheromone blend that differs markedly from the population mean. Further studies are necessary to elucidate the mechanisms of kin- and population-recognition in European beewolves.

The multivariate nested ANOVAs indicate that pheromone composition is affected more strongly by family than subpopulation affiliation on a local scale, whereas the effect of population affiliation on a regional level is larger than the family effect (Table 4.2). These results are consistent with the positive correlation between chemical and geographical distance (Fig. 4.4) and suggest that (1) the subpopulations sampled on the local scale may be connected by a relatively frequent interchange of individuals and might, thus, represent a single population, and (2) small differences in the chemical profile between local subpopulations may add up on a regional scale according to an isolation-by-distance model (Wright 1943). Interestingly, the family effect exceeds the local subpopulation effect, making family differentiation potentially easier for beewolf females than subpopulation discrimination.

Generally, conspecific populations differ genetically only if the gene flow is sufficiently counterbalanced by the divergent forces of genetic drift or natural selection (Futuyma 1998). The reasons for the geographical differentiation in pheromone composition of male European beewolves are not yet known. However, selection pressures might vary among populations and may account for the observed differences. Males in colder regions, for example, may be selected for a greater abundance of pheromone substances with high volatility compared to males from warmer regions. Alternatively, female preferences for pheromone characteristics may vary among populations and cause a divergence of the pheromone composition at different localities.

4.5 CONCLUSIONS

Using GC-MS, we were able to detect differences in the sex pheromone composition of male European beewolves between families as well as among (sub)populations on both a regional and a local scale, with pheromone differentiation being significantly correlated with geographical distance. If female beewolves use this information on kinship and geographical origin contained in the male sex pheromone, they may be able to choose adaptively among potential mates according to the model of optimal outbreeding, thus, avoiding the deleterious effects of both in- and outbreeding by choosing a mate of intermediate genetic distance (Bateson 1983; Shields 1993). Studies considering both in- and outbreeding avoidance in an

integrated model of "optimal outbreeding" are scarce (but see Bateson 1978; Bateson 1980; Palmer & Edmands 2000). The European beewolf constitutes an interesting model system to test for optimal outbreeding in a species with a complex male sex pheromone, and further studies may show whether females indeed use the male pheromone to avoid in- and outbreeding.

4.6 MATERIALS AND METHODS

4.6.1 Insects and sampling

In 2004, four subpopulations with distances ranging from 3 to 18 km (median = 10 km) were sampled (data set 1: local scale; subpopulations: Würzburg, Biocenter, Germany, (49°46´47´´N, 09°58´11´´E), Würzburg, City, Germany, (49°47´56´´N, 09°55´38´´E), Veitshöchheim, Germany, (49°48´20´´N, 09°53´22´´E), Retzbach, Germany, (49°54´55´´N, 09°49´16´´E)). Additionally, specimens from two distant populations in Germany were sampled to allow the combination with data set 2 for the analysis of the association between chemical and geographical distance (Schweinfurt, Germany, (50°03´00´´N, 10°14´00´´E), and Düsseldorf, Germany, (51°11´13´´N, 6°48´09´´E)). The distant populations were not included in the analysis of the chemical differentiation between the subpopulations. In 2005, we were able to sample five populations with distances ranging from 31 to 911 km (median = 490 km) (data set 2: regional scale; populations: Würzburg, Germany, (49°46´47´´N, 09°58´11´´E), Schweinfurt, Germany, (50°03´00´´N, 10°14´00´´E), Düsseldorf, Germany, (51°11´13´´N, 6°48´09´´E), Vizzola Ticino near Milano, Italy, (45°37´35´´N, 08°42´14´´E), and Puttenham near London, UK, (51°13'20"N, 0°40'02"W)).

Female European beewolves were collected at each of the locations given above. They were transferred to laboratory cages at the University of Würzburg and reared after Strohm (1995). Cocoons with larvae of the F1 generation were placed individually in Eppendorf® tubes and kept in boxes with moist sand at 10°C for four to nine months of overwintering. Cocoons were then transferred to warm conditions (cycles of 12 hours at 25°C and 12 hours at 22°C) and adult beewolves emerged four to six weeks later. Emerging males were marked individually with up to three spots of acrylic paint on the dorsal side of the thorax and were allowed to fly in a climate chamber (2.5 x 1.8 x 2.1 m in size) with 12h light/dark cycles at 25°C/20°C and

provided with honey *ad libitum*. Since very young males have been shown to considerably differ in amount and composition of the pheromone (Kaltenpoth & Strohm 2006) males were all caught at an age of 12-17 days and kept in small polystyrol vials (height: 80 mm; diameter: 35 mm) with moist sand and a drop of honey for two days to allow the pheromone glands to be replenished. After anesthetizing the males with CO_2, they were killed by freezing and kept frozen (at –20°C) until extraction of the pheromone and GC-MS analysis.

Overall, 393 males were used for the analysis (Data set 1: Würzburg, Biocenter, Germany: 54, Würzburg, City, Germany: 76, Veitshöchheim, Germany: 35, Retzbach, Germany: 26, Schweinfurt, Germany: 57, and Düsseldorf, Germany: 12; Data set 2: Würzburg, Germany: 46, Schweinfurt, Germany: 26, Düsseldorf, Germany: 8, Vizzola Ticino, Italy: 24, and Puttenham, UK: 29).

4.6.2 Gas chromatography – mass spectrometry

Frozen males were decapitated and their heads were cut at both sides to open up the postpharyngeal gland, which is the storage organ of the male sex pheromone (Herzner et al. 2007; Kroiss et al. 2006). Heads were placed individually in glass vials (1.5 ml), and 20 µl of a 1g/l solution of octadecane in hexane (equivalent to a final amount of 20 µg of octadecane) was added as an internal standard to each vial to allow quantification of the pheromone. The heads were then submerged in approximately 1 ml distilled hexane and chemicals were extracted for four hours.

After extraction, samples were analyzed immediately by coupled capillary gas chromatography-mass spectrometry (GC-MS) with an Agilent 6890N Series gas chromatograph (Agilent Technologies, Böblingen, Germany) coupled to an Agilent 5973 inert mass selective detector. The two data sets were run on the same GC-MS device, but with different capillary columns and slightly different temperature programs. GC-MS set-up 1 (data set 1): The GC was equipped with a HP-5 fused silica capillary column (J&W, 30 m x 0.32 mm ID; df = 0.25µm; temperature program: from 60°C to 300°C at 5°C/min, held constant for 1 min at 60°C and for 10 min at 300°C). GC-MS set-up 2 (data set 2): The GC was equipped with a RH-5ms+ fused silica capillary column (J&W, 30 m x 0.25 mm ID; df = 0.25µm; temperature program: from 120°C to 300°C at 3°C/min, held constant for 1 min at 120°C and for 1 min at 300°C).

Helium was used as the carrier gas with a constant flow of 1 ml/min. A split/splitless injector was used (250°C) with the purge valve opened after 60 sec. The electron impact mass spectra (EI-MS) were recorded with an ionization voltage of 70 eV, a source temperature of 230°C and an interface temperature of 315°C. Since preliminary analyses had revealed that the total amount of chemicals in the sample has an effect on the detection and quantification of certain components, samples in which the pheromone concentration was either too high or too low were rerun after adjusting the pheromone concentration by addition or evaporation of hexane.

4.6.3 Statistical analysis

4.6.3.1 Pheromone amount and composition.

In the pheromone extracts, 25 components could be reliably detected in all samples, and their peaks were manually integrated with MSD ChemStation software (Agilent Technologies). The substances were identified by comparison of mass spectra and retention times with earlier analyses (Kroiss et al. 2006; Schmitt et al. 2003). Not all substances described as components of the pheromone by Kroiss et al. (Kroiss et al. 2006) could be detected due to the low concentrations of the pheromone extracted from single males. Using the octadecane peak as an internal standard, the total amount of pheromone was calculated and then log10-transformed to obtain normally distributed data for statistical analysis. The log10-transformed absolute amounts of pheromone were compared among populations by ANOVAs. SPSS 13.0 software was used for the calculations. The relative amounts of the 20 pheromone components were calculated (peak area / total peak area). Because the relative amounts constitute compositional data, they were transformed to logcontrasts prior to analysis (Aitchison 1986).

4.6.3.2 Chemical dimorphism.

A histogram with the Aitchison-transformed proportion of heptacosene revealed a clearly bimodal distribution without any overlap and, thus, allowed us to unambiguously assign males to the two different morphs (value<0.55: C_{25}-type; value≥0.55: C_{25}/C_{27}-type; see (Kroiss et al. 2006) for a detailed description of the dimorphism, figures showing chromatograms of both morphs and a histogram with the frequency distribution of the

proportion of heptacosene in a population of 45 males). Chance variations in the proportion of C_{25}- and C_{25}/C_{27}-type males among families and among populations can greatly influence the outcome of statistical analyses on the chemical differentiation. Therefore, all of the analyses were performed on C_{25}- and C_{25}/C_{27}-type individuals combined as well as on C_{25}-type individuals only. Thus, by excluding the C_{25}/C_{27}-type males from the analysis, we could make sure that statistical differences between populations or families are due to the overall chemical profile rather than just the frequency of the two distinct chemical morphs. The sample size of C_{25}/C_{27}-type males was too small for a reasonable analysis excluding the C_{25}-type individuals.

4.6.3.3 Population differentiation.

The number of describing variables was reduced by principal components analyses (PCA, Aitchison-transformed relative amounts of pheromone components as variables, varimax rotation, factor extraction: eigenvalues > 0.8). The extracted PCA factors were used for DAs to test whether males of different populations can be separated based on their pheromone profiles. The number of PCA factors used for the DAs was restricted to a maximum of N/6 (N = total number of males in the analysis) to avoid an excess of variables that may increase the risk of false-positive results. This procedure is conservative with regard to the hypotheses tested. A PCA and a DA was conducted for each of the two data sets, respectively. For PCA and DA, data set 1 was restricted to the local scale, including only subpopulations in close spatial vicinity (Würzburg, Biocenter, Würzburg, City, Veitshöchheim, Retzbach: maximum distance: 18km) to exclude regional effects on the outcome of the DA.

4.6.3.4 Family differentiation.

To determine whether families within populations can be separated on the basis of the chemical profile, PCA and DA were conducted as described above for each population for which at least three families with five or more brothers were available. SPSS 13.0 software was used for the principal components and discriminant analyses.

4.6.3.5 Relative effects of family and population affiliation on pheromone composition.

We conducted a multivariate nested ANOVA on the Aitchison-transformed relative peak areas with family membership as a nested factor within populations to assess the relative effects of family and population affiliation on the pheromone composition. For each data set,

two ANOVAs were computed, one including both C_{25}- and C_{25}/C_{27}-type males and the other one with C_{25}-type individuals only. For every pheromone peak, the proportion of variance explained by the two factors was estimated by partial η^2-values (Cohen 1973; Keppel 1991; Olejnik & Algina 2003). To assess the relative effects of family and population on the pheromone composition, the η^2-values for both effects were compared over all peaks in paired t-tests after checking for normal distributions using Kolmogorov-Smirnov tests. All tests were computed using SPSS 13.0 software.

4.6.3.6 Association between geographical and chemical distance.

The geographical distances between all sampled populations were calculated from the population coordinates with the DIVA-GIS software (Wilson & Harrison 1983) and subsequently log-transformed. The chemical distances between the populations were calculated as follows: The mean for each of the 20 Aitchison-transformed pheromone components was calculated for all populations. The chemical distance between two given populations x and y was calculated as the Euclidean distance according to the formula $D_{chem}(x,y) = \sqrt{\sum_{i=1}^{n}(x_i - y_i)^2}$ with xi as the mean of pheromone component i of population x. To be able to combine data sets 1 and 2, which differed slightly due to the differences in GC-MS set-ups, chemical distances were normalized. To this end, we assumed that the chemical distances between two populations that were sampled in both data sets were identical and served as a reference. Consequently, we were able to normalize the chemical distances of the two data sets with the distance between Würzburg and Schweinfurt, Würzburg and Düsseldorf, and Schweinfurt and Düsseldorf, respectively. The respective chemical distance was set to 1 in both data sets and all other values were converted to relative chemical distances. The normalizations based on the three different reference distances revealed qualitatively the same results in the following analyses, indicating that the procedure yielded valid results. The relationship between geographical and chemical distances was visualized using a scatter-plot and a linear regression line. We tested for a correlation between the matrices of geographical and chemical distances using a Mantel test that can deal with missing values using the software R 2.3.0 (mantel.test from the ncf package) (Legendre & Legendre 1998; Mantel 1967). P-values were calculated based on 100,000 resamplings. Mantel tests were performed with each dataset separately, and with the normalized combined dataset. All tests were conducted with both C_{25}- and C_{25}/C_{27}-type males and with C_{25}-type-males only to exclude effects of different relative frequencies of both chemo-types across populations.

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CHAPTER 5

MALE TERRITORIALITY AND MATING SYSTEM IN THE EUROPEAN BEEWOLF *PHILANTHUS TRIANGULUM* (HYMENOPTERA, CRABRONIDAE) – EVIDENCE FOR A "HOTSPOT" LEK POLYGYNY.

in preparation

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5.1 SUMMARY

Males of the European beewolf (*Philanthus triangulum*), a solitary digger wasp, show extensive territorial behavior. They establish small territories, which do not contain any resources essential to females. Territories are intensively scent-marked with a specific pheromone to attract receptive females. Some males show neither territoriality nor scent-marking behavior and apply an alternative "satellite" mating tactic. Using spatial statistics, we show that beewolf territories are a clumped in space both with respect to other male territories and more importantly with respect to female nesting sites. Additionally, the proportion of days a territory is occupied by a male is correlated with the number of female nests in a 10 m radius. Therefore, we conclude that the European beewolf exhibits a "hotspot lek polygyny" with female nesting sites constituting "hotspots" for lek formation.

5.2 INTRODUCTION

The mating system of a given species depends on the distribution of females (or more generally of the sex with limited availability) in time and space and the consequent potential for monopolizing females by males (Emlen & Oring 1977). In species with a low potential to control exclusive access to females by guarding resources essential to females or the females themselves, a male dominance polygyny is likely to evolve. In this case, males sort out their

relative position of dominance and they may cluster in a mating arena called “lek”, where they occupy small territories, which do not contain any resources essential to females. Females visit this arena to choose a mate. Males usually use optical, acoustical, or chemical signals as well as physical fighting to establish a hierarchy and/or to attract females (Bradbury 1977; Bradbury 1981; Höglund & Alatalo 1995; Shelly & Whittier 1997; Wiley 1974). Males in a male dominance polygyny by definition do not provide any paternal care.
In insects, mating systems vary considerably according to the prevailing ecological conditions. In particular, there are a number of well-known examples of female defense and lek polygyny (for a review see Shelly & Whittier 1997). In territorial species, much emphasis has been placed on the factors affecting fighting success among males (size, age, or energy reserves, but also arbitrary criteria like territory ownership; e.g. Coelho & Holliday 2001; Kemp & Alcock 2003; size, age, or energy reserves, but also arbitrary criteria like territory ownership; e.g. Kemp *et al.* 2006); see also (Kemp *et al.* 2006; Takeuchi 2006). Less attention has been drawn to the peculiarity of species, where males display by use of pheromones to attract females,
Males of the digger wasps genus *Philanthus* (about 135 species) are known to establish and scent mark small territories that do not contain any resources (Evans & O´Neill 1988). Males possess a clypeal brush and ventral abdominal hairs that serve to spread the pheromone that is secreted from a postpharyngeal gland (PPG) (Alcock 1974; Alcock 1975; Alcock 1975; Kroiss *et al.* 2006) onto the territory substrate. Merely one species, the North-American *P. albopilosus*, is not territorial, has at least reduced cephalic gland, and lacks the clypeal brush (Evans & O´Neill 1988). In all other *Philanthus* species, territoriality is a mating tactic shown at least by a part of the male population (Alcock 1975; Evans 1982; Evans & O'Neill 1978; Gwynne 1980; Gwynne 1978; O'Neill 1979; O'Neill 1983; O´Neill 1983; Simon Thomas & Poorter 1972; Strohm & Lechner 2000).

Because males are most likely limited with regard to the production of the presumably costly pheromone, males might bypass the production of the pheromone either by fighting for and, in the case of success, occupying an alien territory or by hiding inside the territory to intercept matings. This so called satellite strategy is well known in several species (Evans & O'Neill 1988). Although matings with satellite males have been observed, possession of a territory seems to result in a higher mating success. Whereas several studies report on the territorial behaviour and mating system of North American species (for a review see Evans & O'Neill 1988) there is only little information about the mating system of the European beewolf *P. triangulum* (Simon-Thomas & Poorter 1972; Strohm & Lechner 2000). In particular it has not been clearly shown that the territories constitute a lek situation and whether the scent marking functions to attract females.

If the male territories constitute a lek they should not contain any resources that might be important for females. Therefore, we characterize male territories with regard to possible resources. Leks might be established either around a very attractive male (hot-shot; Hovi *et al.* 1997; hot-shot; Partecke *et al.* 2002) or at places of high female density (hot spot). Here, we assess the hypothesis that the territories of male European beewolves cluster around female nesting sites and might, thus, represent "hot-spot" leks (Schroeder & White 1993; Westcott 1994).

The secretion that is applied to the territory by males might have three more or less plausible (non mutually exclusive) functions: it might (1) attract conspecifics and represent an aggregation pheromone, (2) deter intruders and represent a territorial pheromone, and (3) attract females and represent a sex pheromone. Alternative 1 is the least likely, since there seems to be no reason for males to attract conspecifics as is for example the case in scolytid beetles that reproduce in tree bark and their success is higher when acting collectively (for a review see Raffa 2001). Alternative 2 implies that territories with more males are more attractive for females and the prediction follows that males should not fight intruders and that additional males might also scent mark to attract even more males. Alternative 3 seems most plausible and is already supported by data from North American species (Evans & O'Neill 1988). If the secretion is a sex pheromone and functions to attract females, males should defend their territories against other males and they should reduce scent marking when other males are in then vicinity of their territory since these males might intercept approaching females and copulate while the owner is occupied with scent marking.

5.3 MATERIALS AND METHODS

5.3.1 Study sites and observation methods

Male behavior was investigated at two field sites. The first site was a sand dune near Haidt, Germany (Ha; 49°46'44"N, 10°15'32"E). The second site was a commercial sand pit in Randersacker, Germany (Ra; 49°44'42"N, 9°59'26"E). The two study sites were intensively scored for male territories. A spot was defined as a territory, when a male was seen scent marking there or a male was encountered there on two consecutive days showing typical perching or scent marking behavior.

At the sand dune (Ha) the steep slope was covered with numerous holes and it was not possible to decide whether a burrow was in use. Therefore, at this site, the activity of females and males was determined by counting the number of individuals that occurred (sitting or flying) in sectors of 2 m during 3 minutes of observation (the whole steep slope was 120 m long). The counting was repeated on different days (5 times for females and 4 times for males). For analysis, means of these values were used.
In Ra, the location of female nests and male territories were determined to the nearest 5 cm in a study area of 107 x 18 m and translated to a map of the study area. The surrounding area (within a radius of about 150 m) was scanned for further nests and territories, but no additional nests or territories were discovered. Territories were marked with numbers on adhesive tape that was glued onto vegetation within the territory. The number of active female nests within a radius of 10 m around a territory was determined. This was accomplished by counting the characteristic entrances and mounds of female burrows.

5.3.2 Satellite behavior

At Ra, the behavior of satellite males was observed and described in detail. Additionally, the frequency of marking runs of the territory owner was recorded and related to the presence and number of satellite males in the territory.

5.3.4 Semi-field cages and temporal patterns of scent marking

Male scent-marking behavior and particularly its diurnal pattern were investigated in large flight cages near the Biocenter of the University of Würzburg. Flight cages were 5.0 x 5.0 x 2.0 meters in size and contained nine artificial territories consisting of a wooden central bar (1 m high, 2 cm diameter) and 10 vertically attached wooden rods (length: 20 cm, 0.3 cm diameter) in the upper half of the central bar. 15 to 20 males and several females from a laboratory population were introduced into the flight cages and allowed to establish territories for two days. Marking behavior was subsequently observed for several days from 08:30 a.m. to 05:30 p.m.. Up to three males were observed simultaneously for 30 minutes and the total marking time within this period was recorded. 56 males were observed 1 to 13 times for 30 minutes each (mean: 7.8; total of 510 observations). For analysis, mean marking time per 30 minutes of observation was calculated for each 30 minute interval beginning at

8:30 a.m. and ending at 5:30 p.m.. Scent-marking activity was analyzed using a polynomial regression (BIAS for Windows, version 8.2, epsilon-Verlag GbR, H. Ackermann, Frankfurt/ Main, Germany).

5.3.5 Spatial statistical analysis

The spatial distribution of male territories and female nests and their spatial relationship were analyzed in detail for the Ra site. The spatial pattern of male territories and female nests and the incidence of spatial co-occurrence of males and females were investigated using Ripley's L-function, a square root transformation of Ripley's K-function (Diggle 1979; Ripley 1981; Wiegand & Moloney 2004). This function is a second-order statistic based on the distribution of distances of pairs of points and describes the spatial correlation of point patterns. The square root transformation removes scale dependence and stabilizes the variance. Monovariate L-function was used to assess, whether male territories show a random, clumped, or regular distribution. Bivariate L-function was used to assess the pattern of male territories with respect to female nests.

In bivariate point pattern analyses, the choice of an adequate null model is crucial (Wiegand & Moloney 2004). We chose the model of an "antecedent condition", which assumes that the two types of points were created in sequence. This means that creation of pattern 1 (here: female nests) occurred independently of pattern 2 (because it did not yet exist) but creation of pattern 2 (here: male territories) may be influenced by the presence of points of pattern 1. Under such an antecedent condition, the null model needs to conserve the locations of pattern 1 whereas a null model for pattern 2 needs to be formulated. We applied a null model of "complete spatial randomness" (CSR) for pattern 2. Such a null model of an antecedent condition is appropriate for the analysis of the spatial co-variation of female nests and male territories, since female nests were observed to be present before the establishment of male territories. The 95% confidence intervals for both monovariate and bivariate analyses were computed from 99 Monte Carlo simulations (Birkhofer et al. 2006; Stoyan & Stoyan 1994; Wiegand et al. 2000). L11 (monovariate analysis) and L12 (bivariate analysis) values within the 95% confidence envelope indicate a random association. Values above the confidence envelope reveal significant association between the point patterns and values below the confidence envelope indicate a significant segregation of the point patterns. All analyses were performed using the Programita software (freely available under

http://www.oesa.ufz.de/towi/towi_programita.html), a software program written by Wiegand and Moloney (Wiegand & Moloney 2004).

5.4 RESULTS

5.4.1 Territories

The territories found in the field did not contain any resources that might be of value for females. Often they consisted of dry plants or even bare ground. Sometimes they were found on lower branches of pines, plum trees or broom. However, there were no flowers in the territories that might either serve as nectar sources for the females or for the females' prey, honeybees. Furthermore, at several sites female nests were located within a male territory. When such a female came back from a hunting trip with a bee, territorial males often pounced on them and tried to copulate. However in all cases (n = 29) females did not allow the males to copulate but bent their abdomen downwards and either tried to enter the burrow or even flew away with the bee. This suggests that copulation is under control of the considerably larger females. Despite a total of several 100 h of observations in the nesting area, males were never seen patrolling the emergence sites, or nests of females, or flower patches that were known as potential female hunting sites. Males sometimes encountered females when feeding on flowers. However, they never made any attempts to copulate.

5.4.2 Scent-marking

In the field, the timing of male territorial behavior depended on weather conditions and started at about 10:00 a.m.. On very hot days males were only active until 3:00 p.m.. Then they were seen feeding on flowers. Territorial activity started with marking runs on the vegetation. When alone in the territory, males often perched downwind to the scent marked area at the edge of the territory in an upright position with their fore-body characteristically raised from the substrate and their forelegs closely attracted to the thorax.

In the flight cages, marking runs occurred from 8:45 a.m. to 4:45 p.m.. Maximum marking rate was observed in the early morning and in early afternoon with a slight decrease in the noontime (polynomial regression, $p < 0.001$, $R = 0.919$, Fig. 5.1).

5.4.3 Satellites and non-territorial behavior

A part of the male population did not establish territories and were never observed to scent mark. Some of these males were, however, observed in or in the vicinity of particular territories and seemingly tried to hide from the owners (see below). It was never observed, that two males marked in the same territory simultaneously or at the same day. Thus, territories are exclusive at least with regard to the marking behaviour. Furthermore, some males that have been caught and marked while feeding on flowers (mainly *Cirsium arvense* and *Solidago canadensis*) were never seen either scent marking or near territories.

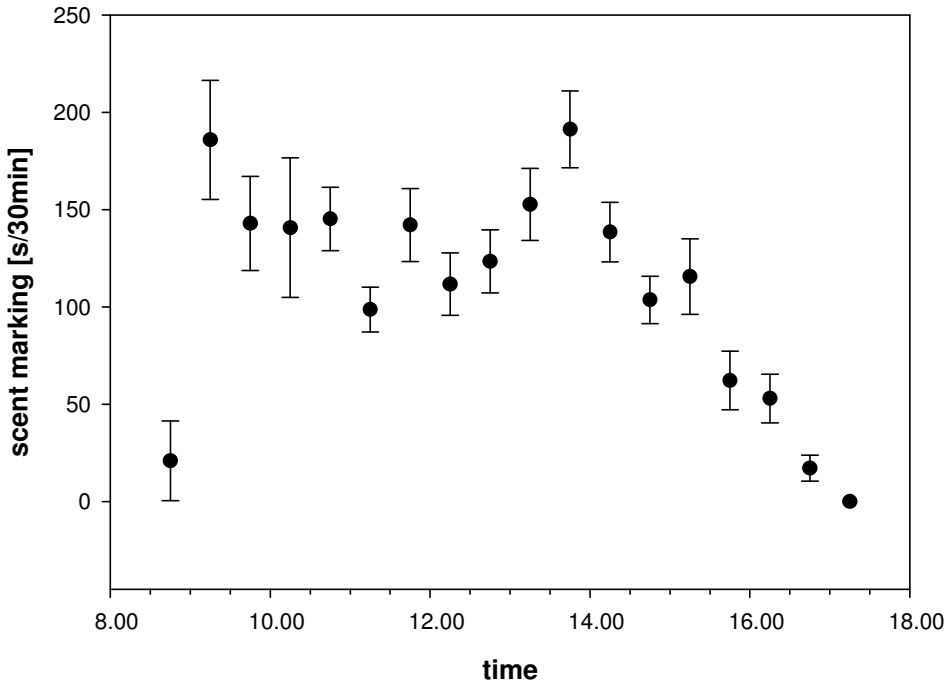

Fig. 5.1: Diurnal pattern of scent-marking of male European beewolves (mean ± SEM; polynomial regression, $p < 0.001$, $R = 0.919$).

5.4.4 Territory defense

Intrusion by other males in a territory resulted in pursuit flights. Single bouts of such fights lasted for minutes. However, a territory owner could be seen fighting repeatedly with the same intruder over hours. During fights males swinged laterally to and fro with an amplitude of about 20 - 40 cm. From time to time one opponent took a position below the other male and suddenly pounced upwards seemingly trying to butt his rival. All flight manoeuvres were performed with high velocity. There were no grabbling or prolonged phases of physical

contact. Flight combats were interrupted when one male – mostly the intruder – sat down somewhere in or near the territory. Contrary to the resident male, intruders did not perch in an upright position but seemed to nestle their body against the substrate. Territory owners then often searched for the intruder and pounced on him. Then the zigzagging flight started again.

The frequency of marking runs of the territory owner significantly decreased from 14.6 % of 259 occasions when only the territory owner was present to 6 % of 80 occasions when a second male was in the territory ($chi^2 = 3.91$, $df = 1$, $p < 0.05$). Thus, males mark less frequently if there is an intruder in the territory.

5.4.5 Spatial distribution and localization of male territories

Ha-Site: Territories were mostly located at the ridge of the dune and males scent marked on roots of the pine trees that had been exposed by the sand winning. Here, about one third of the territories encompassed the entrances of female nests. The number of males in a sector was positively correlated with the number of females coming in with a bee in that sector ($r = 0.36$, $n = 60$, $p < 0.001$). Sectors with a territory showed a higher number of females coming in with a bee (3.31 ± 3.07 per 3 min) than other sectors (1.53 ± 1.73 per 3 min; Kolmogoroff-Smirnov-test: $D = -0.42$, $p < 0.01$). Thus, males establish territories at sites of high nest density.

Ra-Site: Beewolf females were nesting in a steep slope of the sand-pit. Male territories were located above and below the slope close to the female nests (Fig. 5.2). Female nests were restricted to the sandy slope and highly clumped in space (Fig. 5.2). Male territories were highly clumped with respect to other male territories at a distance from 5 to 14 m (monovariate Ripley's L: see Fig. 5.3a). Additionally, male territories were highly associated with female nests in a distance of 3 to 12 m, and slightly, but not significantly, segregated on a distance of 1 m (bivariate Ripley's L: see Fig. 5.3b).

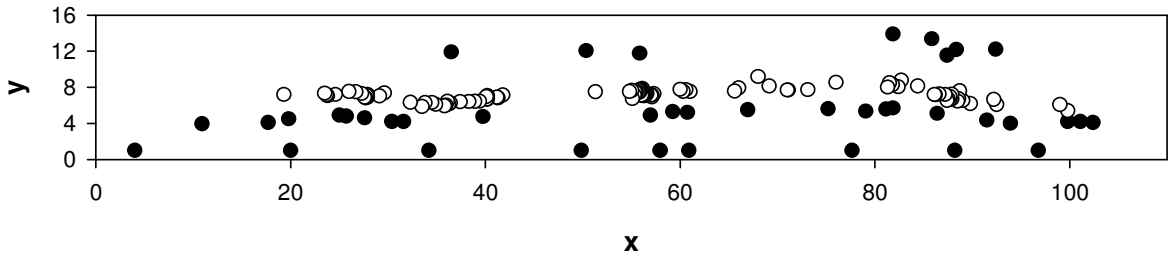

Fig 5.2: Spatial distribution of male territories (filled circles) and female nesting sites (open circles) at the Ra site. Female nests are located in the steep sandy slope of a sandpit and male territories are located above and below the slope.

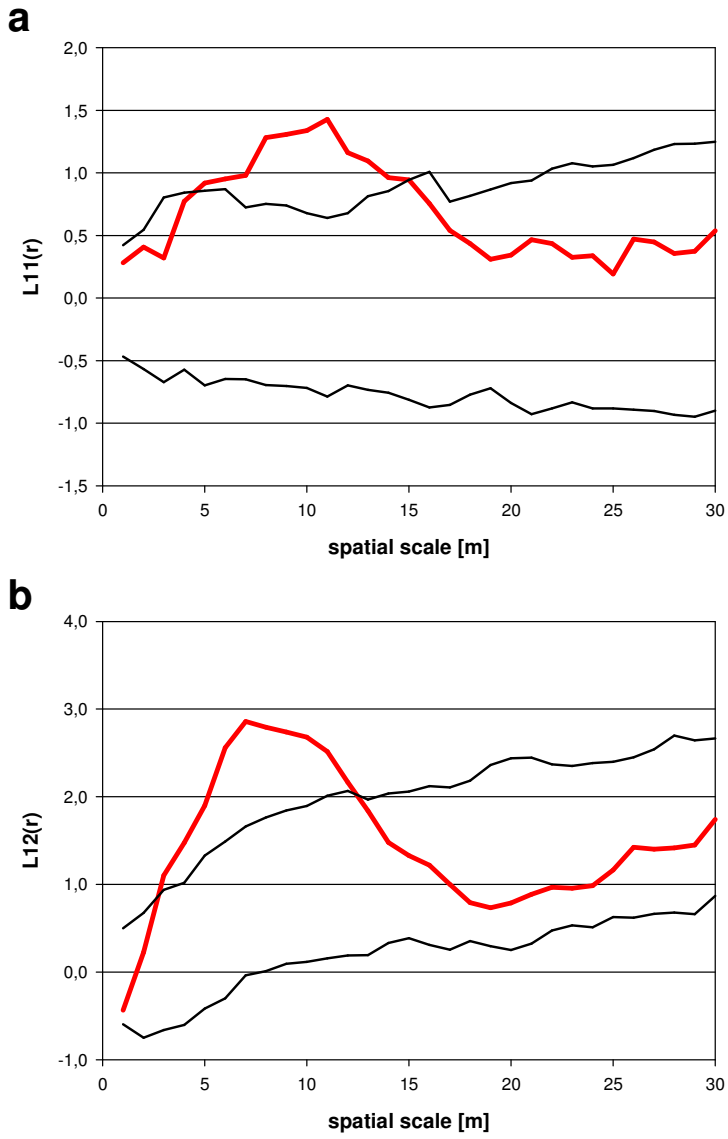

Fig 5.3: Results of Ripley's monovariate (a: male territories) and bivariate (b: male territories with respect to female nests) L(t) analysis. L_{11} and L_{12} values are represented by red lines. Black lines represent 95% confidence intervals generated from 99 Monte Carlo simulations (see text for details). L_{11} and L_{12} values within the confidence envelope indicate a random association, values above the envelope indicate significant association and values below the envelope indicate significant segregation.

Female burrows could be found within the borders of three out of 42 male territories. The number of female burrows in a radius of 10 m around a territory varied from 0 to 25. The number of males in a territory averaged over all observation days correlated with the number of female burrows within the radius of 10 m ($r = 0.662$, $n = 23$, $p = 0.001$, Fig. 5.4). This could be due to an actual higher number of males in the territory or because territories with many burrows in their vicinity were more frequently occupied. The proportion of days a territory was occupied significantly correlated with the number of nearby female burrows ($r = 0.826$, $n = 23$, $p < 0.001$, Fig. 5.5) whereas the actual number of males that were present in a territory was not (averaged over the days when the territory was occupied, $r = 0.13$, $n = 23$, n. s., Fig. 5.6). Thus, the number of nests in the vicinity of a territorial site determines the frequency with which a territory is actually occupied, but not the number of males that are actually present. This result shows that the number of female nests in the vicinity makes certain sites more attractive for establishing and occupying territories.

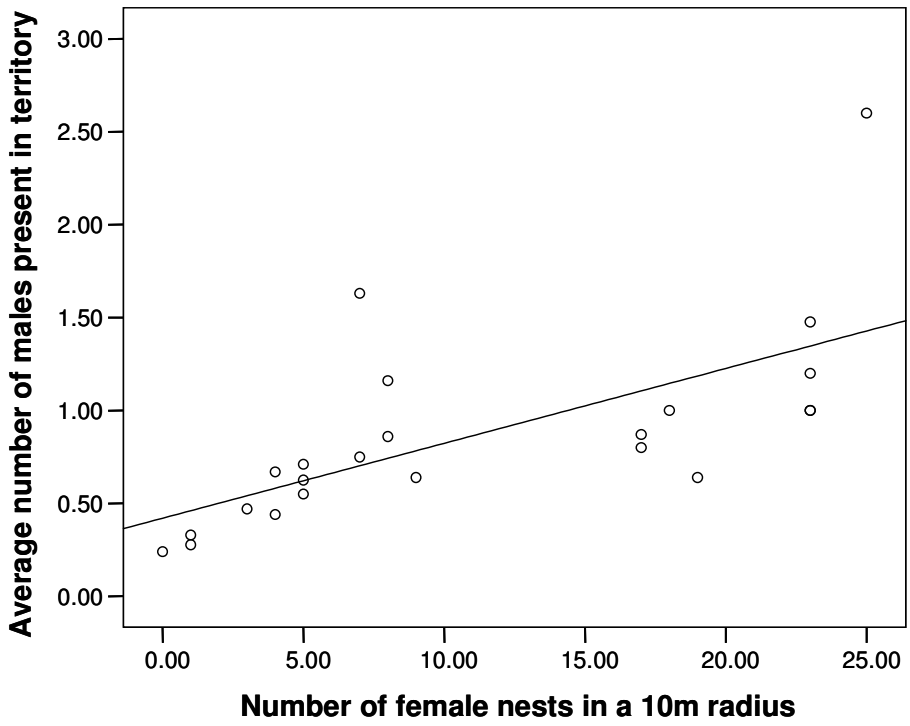

Fig 5.4: Number of males present in a territory over all days as a function of the number of female nests within a radius of 10 m ($R = 0.662$, $N = 23$, $P = 0.001$).

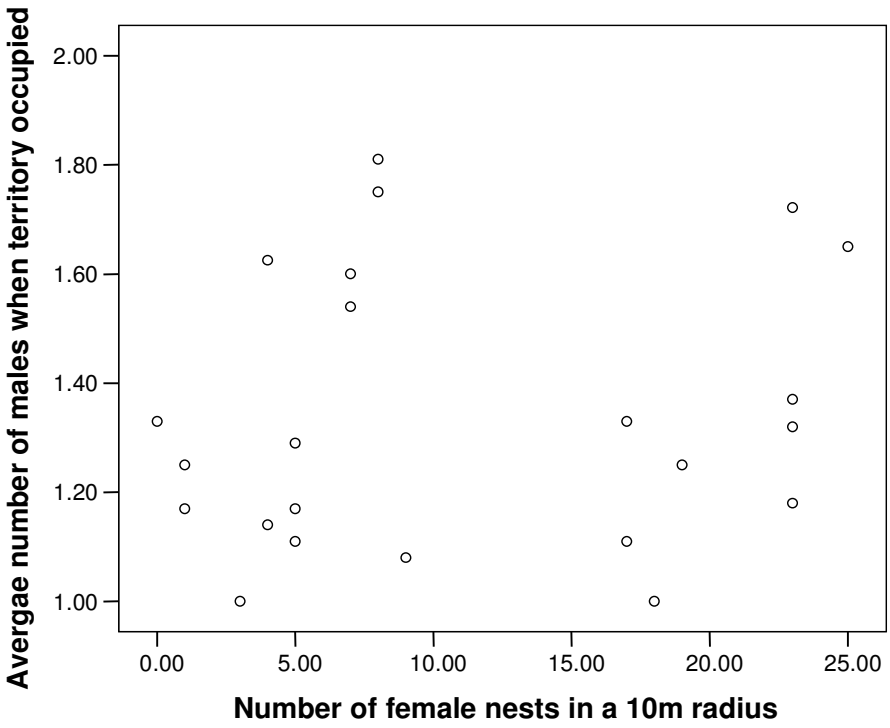

Fig 5.5: Proportion of days a territory was occupied as a function of the number of female nests within a radius of 10 m (R = 0.826, N = 23, P < 0.001).

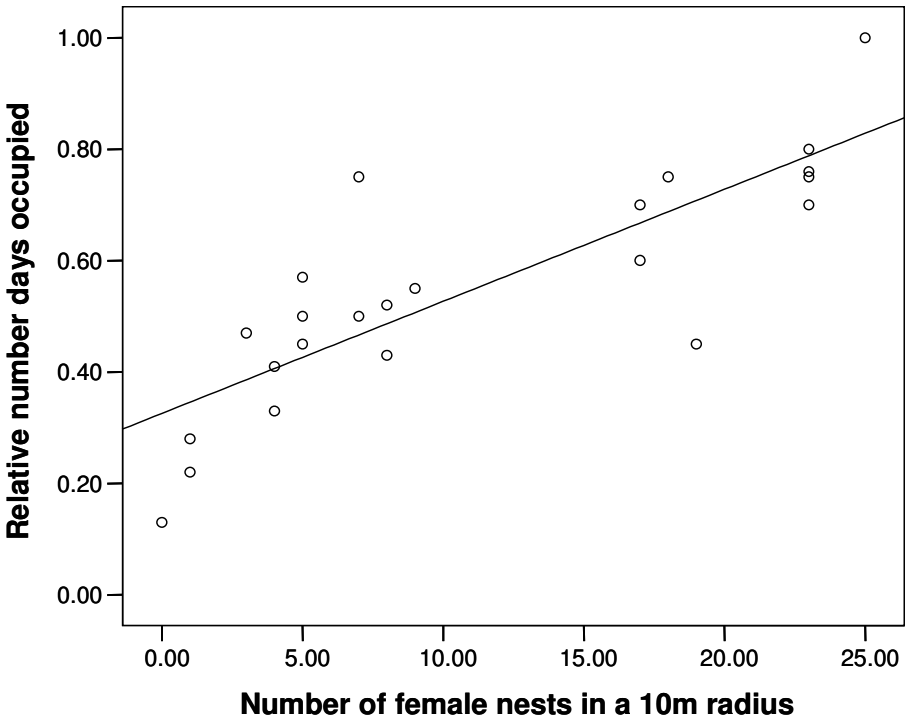

Fig 5.6: Number of males present in a territory, if the territory is occupied as a function of the number of female nests within a radius of 10 m (R = 0.132, N = 23, P = 0.55).

5.5 DISCUSSION

5.5.1 General territoriality

The data presented clearly show that male European beewolves show extensive territorial behavior. Territories are established mostly on dead plants and do not contain any valuable resources for females like flowers. Territories are defended against conspecific males and structures in the territory are intensively scent marked. Male territoriality is a phenomenon known from many vertebrate as well as invertebrate species (for a review see Baker 1983). In insects, a large number of species is known to show male territoriality and the territorial behavior of some species has been studied extensively like in the hilltopping Tarantula hawk wasp Hemipepsis ustulata (Alcock 1981; Alcock & Bailey 1997; Alcock & Carey 1988; Alcock & Kemp 2006; Kemp et al. 2006), in lekking medflies (Field et al. 2002), several butterfly species (Kemp & Wiklund 2001; Kemp & Krockenberger 2004; Kemp et al. 2006; Kemp et al. 2006), and some damselflies (Marden & Rollins 1994; Marden & Waage 1990). By expelling conspecific males from their territory, territory owners usually gain predominant access to receptive females (Brown & Orians 1970).

5.5.2 Alternative mating tactic – satellites

Apart from territorial males, we found non-territorial males called “satellites” in *P. triangulum*. They either try to fight for a territory and eject the former owner or (mostly) sneak into a territory to intercept matings with approaching females. Different behavioral mating tactics and strategies are known from a variety of vertebrate systems like certain toads, where small males cluster around large calling males (e.g. Arak 1988) or in some lek-forming birds (Johnsgard 1994; see also Brockmann 2001; Dominey 1984). Alternative mating strategies are also known from a set of insects like the bee Centris pallida, where large males dig for freshly emerging females and small males hover in the vicinity of the emerging site to mate females missed by the “diggers” (Alcock et al. 1977; see also Paxton 2005). Alternative mating strategies are also known in resource defense polygynies as in the case of Panorpa Scorpionflies (Sauer et al. 1998; Thornhill & Alcock 1983). In most cases the respective strategy is related to somatic features like size and small or inferior males usually adopt a non-territorial and mostly less effective strategy. In other systems, males using

alternative mating strategies even exhibit discrete phenotypic features like horns as in some Scarabid beetle species (Moczek & Emlen 2000) or have a completely different morphology like males in certain fig-wasps (Cook et al. 1997; Cook & West 2005) or ants (Anderson et al. 2003; Cremer & Heinze 2002).

In several species of *Philanthus*, territory owners engage in prolonged fights with intruders that might take the form of zig-zagging aerial combats or might include grabbling and head clashing. While size seems to be the decisive factor for the outcome of territorial disputes in most species(O'Neill 1983; O'Neill 1983), in the European beewolf, *P. triangulum*, size is not the crucial criterion for male territorial performance (Strohm & Lechner 2000; Strohm and Fregin, unpublished data). Also measurements of physiological performance (e.g. flight muscle ratio, wingload; Strohm et al., unpublished data) do not seem to influence the outcome of territorial disputes. Potentially, an arbitrary criterion like territory ownership might be used for the settlement of territorial conflicts. A strong limitation (i.e. high costs) in the production of the pheromone would result in a high motivation of a territory owner to defend the own territory and pheromone investment. However, satellite males might be inferior compared to territorial males either in their ability to produce the sex-pheromone or to defend a territory successfully. Alternatively, potential territories might heavily differ in their quality (e.g. in the number of females passing by the territory), so that it may be a better tactic to be a satellite in a good territory than to be an owner of a bad territory. A single male might also adopt a mixed strategy consisting of territorial and non-territorial behavioral tactics depending on its current (energetic) condition, potentially in the progress of life. Territorial behavior should only be selected, if the reproductive success arising from the territoriality outweighs the costs arising from territory establishment and defense (Emlen & Oring 1977). Reciprocally, the mating success of territorial males should be higher than the success of satellites. Nevertheless, a low mating success is better than no success at all and males should be selected for the application of – even relatively unsuccessful – alternative mating strategies in a sense of “making the best of a bad job”.

Apart from territorial and satellite males, we found a third group of males, which has never been observed showing territorial or satellite behavior, but has only been seen feeding on flowers. Although we can not exclude, that these males had territories in a distance that were not covered in the study (i.e. more than 100 m away from the female nests), this third group has the lowest mating success, since copulations have been observed exclusively to happen or at least start in male territories. Such a skew in the mating success as outlined here is not unusual in polygynous species, especially in systems exhibiting a lek polygyny (Gould & Gould 1997).

5.5.3 Marking pheromone

All *Philanthus* species except for the North American species *P. albopillosus* are known to produce large quantities of a species-specific multi-component male pheromone. This pheromone could in principle function to 1) attract other males (aggregation pheromone), 2) deter intruders, and 3) attract females (sex-pheromone). If the pheromone functions to attract other males, then territory owners should not fight with them. But obviously that is what they do. Territories treated with the pheromone are defended against other males, which in turn seem to be attracted to the territory by the windborne pheromone to "cheat" and to intercept matings as a satellite. Thus, only the female attraction function seems to be plausible. Unfortunately, it has not yet been possible to verify female attraction of the pheromone in bioassays due to the complex behavior of beewolves. Likewise, some details of the role of the pheromone are not known, in particular for how long it volatizes from the substrate. The frequency of marking runs suggests that it has to be renewed after a couple of minutes under good weather conditions with low wind velocities. Males can be seen hovering downwind from the twigs that have been scent marked (Kroiss et al., unpubl. data), presumingly sensing the strength of the odor. Sometimes territory owners flew to nearby territories (Kroiss et al., unpubl. data), possibly to assess the pheromone quantity in the neighboring territories and not to fall behind and be outcompeted by their pheromone.

5.5.4 Mating system of the European beewolf

Territories of beewolf males do not contain any resources like flowers. Therefore we can exclude a "resource defense polygyny" as the mating system of the European beewolf. Similarly, the mating system is no "female defense polygyny" though territories contained female nests in some cases. However, males did not attain mating opportunities with these females since these refuse mating attempts and rather approach territories when receptive. Territories of *P. triangulum* males are clumped in space both with regard to other males but even more clearly with regard to female nesting sites. This spatial analysis suggests that female nesting sites constitute "hotspots" for lek formation. We therefore conclude that the mating system of the European beewolf is a "hotspot lek polygyny", representing a special case of the male dominance polygyny in the sense of Emlen and Oring (1977). The fact that territories are more often occupied, if there are more female nests in the vicinity, supports the "hotspot" model.

Other members of the genus *Philanthus* have been considered to show female defense polygyny or a lek mating system (Evans & O´Neill 1988). According to Gwynne (1980), the bumblebee wolf *P. bicinctus* exhibits a female defense polygyny. However, the evidence not necessarily supports this conclusion. Although there is a correlation of the proportion of time a territory is occupied and the number of female nests in a 3 m radius, this system might also be a lek polygyny. First, beewolf territories are usually much smaller than an area with a 3 m radius, which was scored for female nests in that study. First, beewolf territories are usually much smaller than an area with a 3 m radius, which was scored for female nests in that study. Second, as in *P. triangulum*, copulations also did not occur at the female nests but inside the scent-marked territories. As in *P. triangulum*, the nesting sites of the females most probably constitute “hotspots” where large numbers of females are likely to pass (see Kirkpatrick & Ryan 1991 for the "hotspot"- and alternative hypotheses of lek-evolution; Westcott 1994; see also Beani et al. 2002; Field et al. 2002). Therefore, these “hotspots” are the locations where the leks are established. In *P. bicinctus*, large males may fight for a good position at the female “hotspot”, which might account for the correlation of male size and the number of female nests in a 3 m radius around the territory. Unequivocal examples of female defense polygyny in insects are probably the mating systems of several bee and wasp species, where males monopolize a cluster of females e.g. at emergence sites of females and where copulations take place immediately after emergence (e.g. in the bee *Centris pallida*; Alcock et al. 1977).

While the evolution of lek mating systems is not yet fully understood, there is no doubt that this mating system gives a great opportunity for females to choose among potential mates without elevated costs for searching. The beewolf system offers the opportunity for mate choice according to the properties of the marking-pheromone. Females could either choose by the sheer amount of the pheromone applied to the territory substrate, or according to the interindividual variation in the composition of the complex pheromone blend (Herzner et al. 2006; Kaltenpoth et al. 2007; Kaltenpoth & Strohm 2006), which would allow mate choice according to a model of “good genes” or the model of “best compatibility”.

In conclusion, males of the European beewolf *Philanthus triangulum* show extensive territoriality with the territories clustering at female nesting sites. The mating system is a “lek polygyny”, with the female nesting sites most likely forming “hotspots” for lek formation. Though the factor differentiating territorial from satellite males is not yet known, inferior males might adopt an alternative mating strategy as a satellite in other males’ territories. Even if not yet definitely demonstrated, the beewolf mating system provides ideal conditions for adaptive female mate choice according to the physical performance and/or pheromone characters (amount and composition) of the respective male.

5.6 ACKNOWLEDGEMENTS

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CHAPTER 6

DO MALES OF THE CUCKOO WASP *HEDYCHRUM RUTILANS* (HYMENOPTERA, CHRYSIDIDAE) HAVE A SEX PHEROMONE?

in preparation

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6.1 SUMMARY

While female sex pheromones are known from many insect species, knowledge about the occurrence and chemical characteristics of male sex-pheromones, especially of parasitic Hymenoptera, is largely lacking. In this study, large amounts of a male-specific compound was extracted from the abdomens of males of the cuckoo wasp *Hedychrum rutilans*, a brood parasitoid of the European beewolf, *Philanthus triangulum* (Hymenoptera, Crabronidae). The compound was identified as 5-Hexadecenylbutyrate. The substance is produced in sternal glands and released through channels onto the surface of the fifth and sixth sternite that are normally hidden but bear two conspicuous hair brushes. The pheromone might be secreted by extruding the sternites and dragging the hair brushes over a surface. Since the substance in only found in males a function as a sex pheromone is suggested. Sternal glands also occur in female and male polistine wasps and the potential homology with the cuckoo wasps' sternal glands is discussed.

6.2 INTRODUCTION

Insect sex pheromones and their role for mate attraction and mate choice have been intensively studied over the last decades (Wyatt 2003). While female sex-pheromones have attracted much attention, male sex-pheromones have largely been neglected (Johansson & Jones 2007). Nevertheless, now there is a growing body of evidence that male sex-pheromones are taxonomically widely distributed (e.g. Andersson *et al.* 2007 in butterflies; Haberer *et al.* 2007 in dung beetles; Kock *et al.* 2007 in a scorpionfly; Schmitt *et al.* 2003 in a crabronid wasp) and often play a crucial role for species and mate recognition as well as mate assessment (see Johansson & Jones 2007 for a review). Only recently, the first description of a male sex-pheromone of parasitic Hymenoptera has been published (Ruther *et al.* 2007 for *Nasonia vitripennis*).

Cuckoo wasps (Hymenoptera, Chrysididae) are a family of parasitoids and cleptoparasites of different insect taxa (Kimsey & Bohart 1990). Members of the subfamily Chrysidinae are almost exclusively brood parasitoids of bees or wasps (Kimsey & Bohart 1990). The cuckoo wasp *Hedychrum rutilans* is a specialized brood parasitoid of the European beewolf *Philanthus triangulum* (Hymenoptera, Crabronidae), a digger wasp whose females hunt honeybees as larval food (Kunz 1994). *H. rutilans* females enter the host nest and oviposit on honeybees, which are temporarily stored in the main-burrow of the underground nest (Strohm *et al.* 2001). They have been shown to evade detection by the host by means of chemical mimicry of its cuticular hydrocarbons (Strohm *et al.* 2008). While there is some knowledge about female cuckoo wasps and the interactions with their hosts (Rosenheim 1987; Rosenheim 1989; Strohm *et al.* 2001; Yamada 1987; Yamada 1990) knowledge about male courtship behavior is completely lacking.

Here we describe a conspicuous glandular structure and a release organ of males of the cuckoo wasp *H. rutilans* and analyze the chemical nature of the gland secretion using coupled gas-chromatography – mass spectrometry and derivatization methods. We propose that the secretion is a sex-pheromone that might play a role in the attraction of conspecific females or during courtship.

6.3 MATERIALS AND METHODS

6.3.1 Specimens and sampling of secretion

Males and females of *H. rutilans* were caught at populations in Nürnberg and Retzbach, Franconia, Germany. For chemical analyses, specimens were anaesthetized with CO_2 and killed by freezing at -20°. For morphological analyses see below.

6.3.2 Scanning Electron Microscopy

For scanning electron microscopy (SEM), males were anaesthetized with CO_2 and killed with diethyl ether. The abdomen was cut off and the sternites IV-VII that are usually retracted and therefore not visible were pulled out with pointed forceps. The specimens were fixed in alcoholic Bouin for 3 hours at 4°C followed by dehydration in a graded acetone series. Then they were critical point dried (BAL-TEC CPD 030), sputtered with Pt/Pd (BAL-TEC SCD 005) and examined with a Zeiss DSM 962 digital scanning electron microscope at 15 kV.

6.3.3 Histological investigations

For histological investigations abdomen were fixed in alcoholic Bouin for 3 hours at 4°C, dehydrated in a graded ethanol series and embedded in Poly/Bed® 812 (Polysciences, Eppelheim, Germany). Sections of 4 µm thickness were produced with a diamond knife on a Reichert 2040 Autocut and stained with 1% toluidine blue buffered with 1% Di-sodium-tetraborate in distilled water. Digital photos of the sections were obtained with a Nikon DS-2Mv camera attached to a Zeiss Axioplan microscope.

6.3.4 Sampling and analysis of secretion

Preliminary screening using SPME fibers suggested that large amounts of an unknown substance were found on the surface of the hidden abdominal segments of *H. rutilans* males. Thus, abdomens were cut off from *H. rutilans* males (N = 36) and the hidden abdominal segments were pulled out with pointed forceps. Abdomens were transferred to 1.5 ml glass vials and individually extracted for 4 hours with 300µl of hexane. Hexane (Fluka, Deisendorf, Germany) was distilled and checked for purity by GC-MS prior to use. As an internal standard, 5µl of a 1g/l solution of octadecane in hexane (equivalent to a final amount of 5µg octadecane) was added to each vial to allow absolute quantification of the compound.

6.3.5 Capillary Gas Chromatography – Mass spectrometry (GC-MS) and pheromone identification

Aliquots of the extracts (1µl) were analyzed by coupled GC-MS. The analysis was conducted with an Agilent 6890N Series gas chromatograph (Agilent Technologies, Böblingen, Germany) coupled to an Agilent 5973 inert mass selective detector. The GC was equipped with an RH-5ms+ column (30 m x 0.25 mm ID; df = 0.25 µm; J&W), and the temperature program ramped from 70°C for 1 min to 180°C at 30°C/min, then to 310°C at 5°C/min, hold 5 min. Helium was used as carrier gas (constant flow 1 ml/min). A split/splitless injector was used (250°C) with the purge valve opened after 1 min. Electron impact mass spectra (EI-MS) were recorded with an ionization voltage of 70 eV, a source temperature of 230°C, and an interface temperature of 315°C. MSD ChemStation Software (Agilent Technologies, Palo Alto, CA, USA) for Windows was used for data acquisition. The male-specific compound was identified using GC-MS. The position of the double-bond was determined using dimethyl disulfide (DMDS) derivatization after Dunkelblum and others (1985).

6.4 RESULTS

6.4.1 Scanning Electron Microscopy and histological investigations

Males of *H. rutilans* have three visible abdominal segments (Kimsey & Bohart 1990). In contrast to these very hard and heavily sclerotized segments, the hidden segments are highly flexible and delicate. SEM of the abdominal sternites V and VI revealed dense brushes of hairs on the distal margin of the segments (Fig. 6.1). On sternite V the lateral hairs are longer (about 100 µm) than the central hairs (about 50 µm) (Fig. 6.1). On sternite VI the hairs are up to 200 µm long with only the innermost hairs shorter (Fig. 6.1).

Semithin sections of abdominal sternites V and VI show several class 3 gland cells (according to Noirot & Quennedey 1974) at the intersegmental membranes opposed to the hair brushes (Fig. 6.2). The gland cells are drop-shaped with diameters of about 15-20 µm and are connected to the intersegmental membranes through conducting canals (Fig. 6.2). These canals open out in the cavity which is formed by the sternites and their respective intersegmental membranes when retracted in the abdomen (Fig. 6.2). Females have neither such hair brushes (see also Lorencova 1962), or the adjacent gland cells.

6.4.2 Capillary Gas Chromatography – Mass Spectrometry

GC-MS analysis revealed one sex-specific compound in the abdominal extracts of *H. rutilans* males (Fig. 6.3). Extracts of females did not show any of this substance. Based on the mass spectra of the compound before and after DMDS-derivatization and comparison with the mass spectrum of the synthetic substance, the compound was identified as 5-Hexadecenylbutyrate (Fig. 6.4). A total amount of 9.42 ± 5.57 µg (mean ± SD, N = 36) per individual male was extracted.

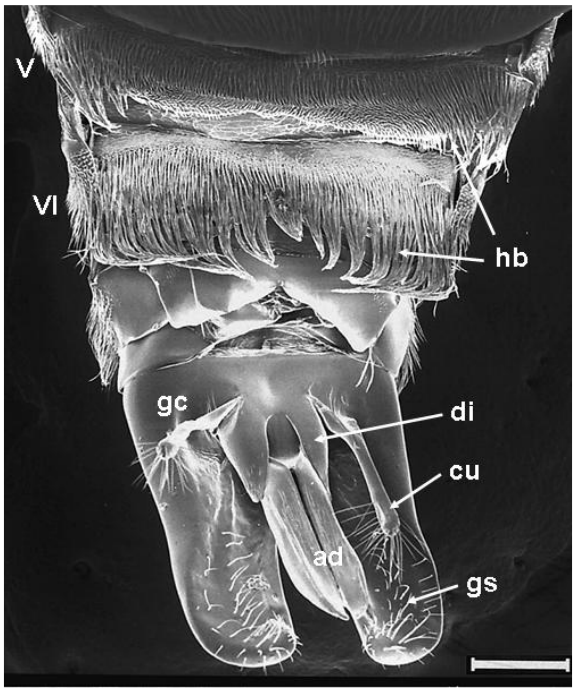

Fig. 6.1: SEM micrograph of the hidden abdominal segments (ventral view) of male *H. rutilans* with hair brushes on the fifth and sixth abdominal sternites (V, VI: abdominal segments; ad: aedeagus; cu: cuspis; di: digitus, gc: gonocoxa; gs: gonostyle; hb: hair brushes; scale bar: 200 µm).

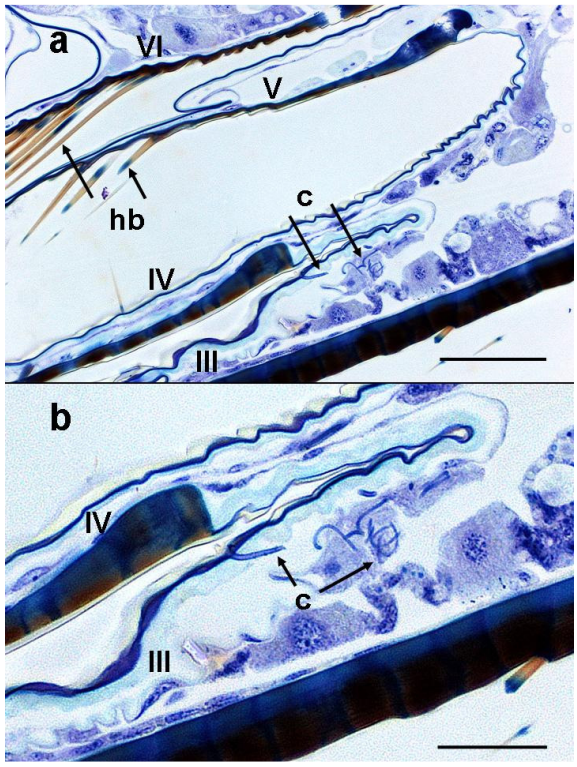

Fig. 6.2: Semithin sagittal section of the abdomen of male *H. rutilans* with sternites III to VI and the intersegmental membranes in their natural position (i.e. retracted). hb: hair brushes on sternites V and VI; c: canal bundles reaching from the gland cells to the intersegmental membrane between the sternites III to VI; III, IV, V, VI: sternites III to VI; scale bars a: 50µm, b: 25µm.

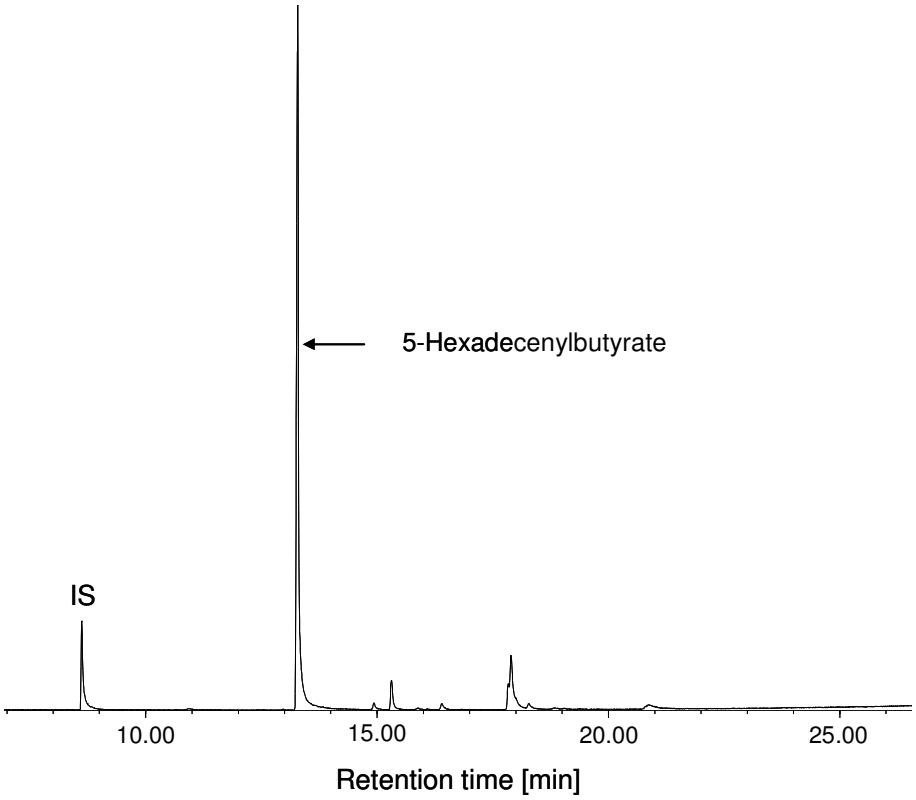

Fig. 6.3: Total ion chromatogram of a hexane extraction of the abdomen of a male *H. rutilans*. The arrow indicates the male sex-pheromone 5-Hexadecenylbutyrate. Small peaks are cuticular hydrocarbons. IS: internal standard (5µg of octadecane).

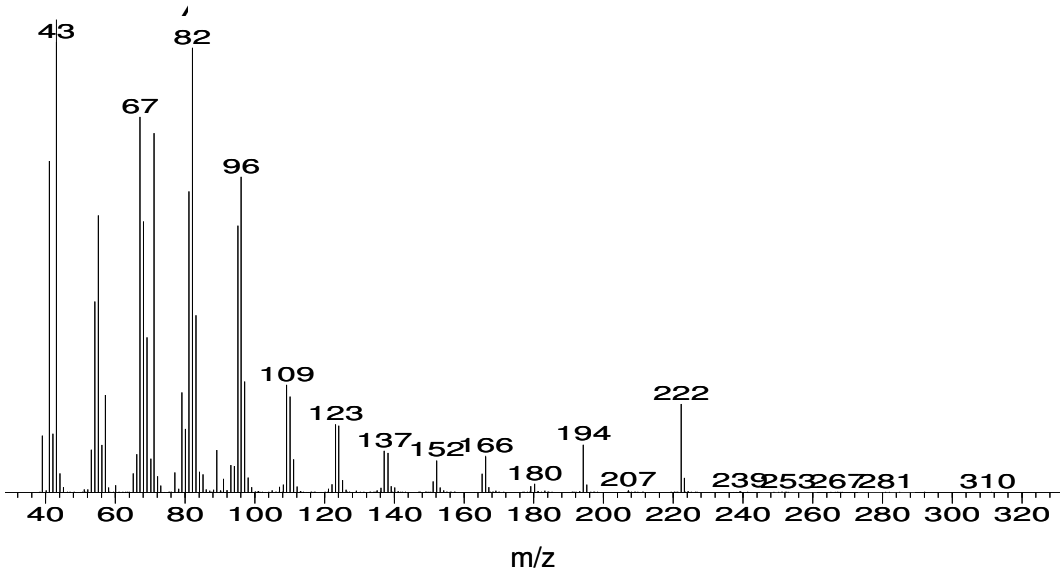

Fig 6.4: Mass-spectrum of 5-Hexadecenylbutyrate, the potential male sex-pheromone of *H. rutilans*.

6.5. DISCUSSION

Our results show that male *H. rutilans* possess sternal glands that are connected to sternal hair brushes that can be exposed by extruding of the abdomen. The glands contain large amounts of a single compound, 5-Hexadecenylbutyrate.

The hidden abdominal segments of chrysidid form the telescope-like genital tube in males and the ovipositor in females (Kimsey & Bohart 1990; Kimsey 1992). On the sternites V and VI we found conspicuous hair brushes. These hair brushes have already been described for males of *H. rutilans* and other members of the genus *Hedychrum* (Lorencova 1973). The hair brushes are not present in *Hedychrum* females (Lorencova 1962) and are also absent in other chrysidid genera.

The location of the class 3 gland cells and the respective conducting canals suggests that the secretory products might be applied onto a surface by the hair brushes in a scent marking process. Possibly, the gland content is secreted by extending their hidden abdominal segments and dragging them over a surface. The hair brushes themselves as well as the space between the retracted sternites and the intersegmental membranes might serve as a reservoir for the secretion.

Sternal glands have been described for some other Hymenoptera. Females of some polistine wasps possess sternal glands on the fifth and/or the sixth abdominal sternite, in cases also associated with hair brushes. Sternal glands on the sixth abdominal sternite have been described by van der Vecht (1968). This so called van der Vecht gland is found particularly in females of independent-founding species (i.e. females found a nest without the aid of workers) of the genera *Polistes*, *Mischocyttarus*, *Parapolybia*, *Belonogaster*, *Ropalidia* (not all species) (Jeanne *et al.* 1983) and the glands have been shown to play an important role in the production of an ant repellent (Jeanne 1970). By abdomen dragging, the secretion of this gland is smeared onto the nest petiole (Keeping 1990) and keeps ants off the nest and the wasp brood. The chemical composition of the gland secretion of *Polistes* wasps has been described by Dani and others (2003) as a mixture of hydrocarbons and a low proportion of fatty acids. The van der Vecht gland has also been described in the giant hornet *Vespa mandarinia japonica* (Hymenoptera, Vespidae) where it is involved in the production of a foraging-site marking pheromone for *en masse* predation (Ono *et al.* 1995).
Sternal glands on the fifth abdominal sternite have been described by Richards (1971; 1978) for female polistine wasps. These Richards' glands usually occur in females of swarm-

founding species and apparently produce a trail pheromone involved in swarming behavior (West-Eberhard 1982). Sometimes, abdomen dragging that might be involved in pheromone application can be observed (Jeanne *et al.* 1983; Jeanne & Post 1982). The chemical composition of the trail pheromone has been identified for *Polybia sericea* as a complex mixture of alkyl and aromatic aldehydes, fatty acids, 3-phenylpropanoic acid, ketones, a macrolactone, a pyranone compound and nerolidol (Clarke *et al.* 1999). Females of swarm-founding species might not need a chemical nest defense against ant predation, since from the beginning of the nest-building phase there are workers that defend the nest against predators.

The occurrence of van der Vecht glands and Richards' glands is highly variable in polistine wasps with sternal glands occurring in the fifth or the sixth abdominal sternite in some species, in both (*Apoica flavissima* and *A. pallida*) and do not occur at all in some genera (Jeanne *et al.* 1983). It seems likely that sternal glands evolved in response to the requirements of communication among nest mates and the mode of nest founding (Jeanne *et al.* 1983; Smith *et al.* 2002).

Abdominal sternal glands have also been described for males of polistine wasps (Downing *et al.* 1985; Landolt & Akre 1979). In males, these glands are believed to produce pheromones used in the context of reproductive behavior (Litte 1979; Post & Jeanne 1982; Post & Jeanne 1983; Post & Jeanne 1983; Turillazzi & Cervo 1982). However, the chemical nature and the exact role of these putative pheromones are not known.

It is not clear whether the sternal glands that we found in *H. rutilans* males are homologous to the van der Vecht and Richards' glands present in male and female polistine wasps. Two criteria of homology, however, seem to be met: the sternal glands of *H. rutilans* males are found at the same site (i.e. the fifth and sixth abdominal sternite) and show similar morphological structures (i.e. class 3 gland cells and hair brushes) as the glands found in polistine wasps. Chrysidid wasps are assumed to represent on e of the most ancient taxa of the aculeate Hymenoptera. The occurrence of sternal glands in this taxon might suggest that these glands are a plesiomorphic character of the aculeates. That other cuckoo wasp species and other major taxa of the aculeates seem to lack sternal glands might either be due to a lack of knowledge or the glands might be easily lost under certain ecological circumstances. The possibility of a rapid loss of sternal glands is exemplified by the pattern of their occurrence within the polistine wasps. Histological as well as chemical investigations of the abdominal sternites of other aculeate wasps (Chrysidoidea, Apoidea, and Vespoidea) (Brothers 1999) might shed light on the evolutionary relationship of sternal glands with the aculeate Hymenoptera.

The compound that we found in comparatively large amounts in the sternal glands of *H. rutilans* males, Hexadecenylbutyrate, has already been described by Francke and others (2000) from cephalic glands of the stingless bees *Trigona hyalinata* and *Tetragonisca angustulata*, by Terzö and others (2005) from the labial gland of male bumblebees, *Bombus sylvarum*, and by García-Rubio and others (2002) from the paracloacal glands of the American Crocodile *Crocodylus acutus*. However in these studies neither the position of the double-bond nor its geometry has been determined. Therefore, this is the first description of 5-Hexadecenylbutyrate in nature.

Due to a lack of information on possible courtship behavior and mating in cuckoo wasps as a whole, the actual function of the sternal glands and of this substance is not yet clear. However, due to the amounts found in males and the volatility of the substance, the most plausible hypothesis is that this substance plays a role in the attraction of females and/or courtship. However, there are no reports on abdomen dragging behavior in cuckoo wasps. Possibly the secretion is applied onto the females body as a part of the courtship prior to copulation. Notably, the secretion might be applied onto the females' ovipositor that bears a number of sensilla that are probably involved in oviposition (E. Strohm, unpublished data). Unfortunately, the mating behavior has not been documented in any cuckoo wasp species. Alternatively, the secretion might serve as a defensive secretion against predators. However, cuckoo wasps are known to adopt a rolled up position when attacked and can hardly be hurt due to their massive cuticle. However, this defensive posture would not allow secreting the gland content. Furthermore, if the secretion has a defensive function it should also occur in females that are probably even more prone to predator attack than males due to their extensive host searching (Strohm *et al.* 2008). Thus, the hypothetical function of the sternal glands and their secretion as a sex pheromone has to be tested by bioassays in the field or in an olfactometer.

6.6. ACKNOWLEDGEMENTS

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CHAPTER 7

HYDROCARBONS IN THE NEST MATERIAL OF A SOLITARY DIGGER WASP REPRESENT A KAIROMONE FOR A SPECIALIZED CUCKOO WASP.

Animal Behaviour: in press

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7.1 SUMMARY

Cuticular hydrocarbons (CHCs) can be found on the cuticle of insects and primarily serve to reduce desiccation. Additionally, they constitute important cues for species, kin, and nestmate recognition. In paper wasps, these CHCs can also be found on the nest material and facilitate nestmate recognition. Here, we analyzed whether the nest material of a solitary diggers wasp, the European beewolf *Philanthus triangulum* (Hymenoptera, Crabronidae) also contains CHCs and whether these might provide cues for the host search of a specialized cuckoo wasp (*Hedychrum rutilans*). We show that ejected nest material contains 3.6 ± 2.1 µg of CHCs per gram. The composition of these nest hydrocarbons (NHCs) is very similar to the CHC composition of the cuticles of beewolf females. In particular, there are two distinct chemomorphs occurring in the European beewolf that can be unambiguously distinguished by means of the NHCs. Because of these large amounts of NHCs, females of the specialized parasitoid *Hedychrum rutilans* (Hymenoptera, Chrysididae) may use these NHCs as kairomones to locate and identify the host nest. Bioassays showed that females of this cuckoo wasp are able to visually recognize nest mounds of their host and are able to gustatory discriminate between host and non-host nest material. Furthermore, in olfactometer assays, *H. rutilans* females stayed significantly longer in the segment supplied with an airflow that passed beewolf nest-material as compared to a control segment. Our results suggest that the beewolf NHCs provide important olfactory cues for a chrysidid parasitoid for host localization and, possibly, optimal host choice.

7.2 INTRODUCTION

The cuticle of insects is covered with cuticular hydrocarbons (CHCs), which primarily serve to reduce water loss through the body surface (Gibbs 1998). Additionally, the CHCs may play a role for species (Howard & Blomquist 2005; Lucas et al. 2005; Singer 1998; Takahashi & Gassa 1995) and – in the case of social insects – nestmate recognition (Dapporto et al. 2006; Gamboa 2004; Greenberg 1979; Ruther et al. 2002; Singer 1998). CHCs can also be extracted from the nest material of social paper wasps and have been shown to facilitate learning of the nest odor and nestmate recognition (Espelie & Hermann 1990; Lorenzi & Caprio 2000; Singer & Espelie 1996). By contrast, there are only few reports about hydrocarbons in the nest material of other insects (e.g. Guedot et al. 2006). This is surprising, since these nest hydrocarbons (NHCs) may play an important role as a pheromone or cue for nest recognition. Furthermore, NHCs may be exploited by parasites or parasitoids to locate and identify host nests, in this case functioning as a kairomone.

The European beewolf *Philanthus triangulum* (Hymenoptera, Crabronidae) is a solitary digger wasp preferentially nesting in sandy soil. Female beewolves hunt exclusively honeybees *(A. mellifera*) as provisions for the larvae. One to six bees are supplied into one brood cell, and the female lays a single egg on one of the bees. Beewolf nests consist of a main burrow that is up to 1m long with up to 34 lateral brood cells that are temporarily connected to the main burrow for provisioning and then carefully closed (Strohm & Linsenmair 1995). During the construction of the burrows and the brood cells, the females excavate and process a large amount of sand. When nesting on a flat or slightly sloped area, sand will accumulate in front of the nest entrance and build up to a characteristic nest mound (see e.g. Evans & O´Neill 1988). If available, beewolves will, however, nest in sandy steep slopes, and in this case, the excavated sand will drop from the nest entrance to the ground and will leave little characteristic markings in front of the nest.

The highly nutritious provisions as well as the developing larvae in the brood cells are threatened by several pathogens and parasitoids. The cuckoo wasp *Hedychrum rutilans* (Hymenoptera, Chrysididae) is a specialized parasitoid of larvae of the European beewolf (Kunz 1994; Strohm et al. 2001; see also chapters 8-10) and it sometimes causes high rates of brood failure in beewolf populations (Simon-Thomas & Simon-Thomas 1972). Females of *H. rutilans* can be observed flying over beewolf nest aggregations and approaching the nest mounds of their hosts. Mostly, the cuckoo wasp female will fly on to another nest. Alternatively, depending on the status and suitability of the nest the cuckoo wasp may enter

the nest to search for stored honeybees and oviposit (see chapter 8). Remote orientation in host searching might be based on optical cues. Near and on the nest mounds, however, cuckoo wasps extensively antennate the surface, suggesting that they use that olfactory and/or gustatory cues for close range orientation. It is not clear what kind of chemical information the nest mound may contain and what the source of these chemicals is. Due to the prolonged usage of a nest by a beewolf female and due to the treatment of prey bees with large amounts of a secretion that is very similar to the CHCs of beewolf females (Herzner & Strohm 2007; Strohm et al. 2008), we hypothesized that the excavated sand might contain traces of the hydrocarbons of the host and that cuckoo wasps use these compounds as kairomones to identify their host nests.

Here we test whether CHCs of the nest owner can be extracted from material of the nest mounds of *P. triangulum* females. Additionally, using bioassays we investigate whether *H. rutilans* females are attracted by a) visual cues during remote searching and b) substances contained in the excavated nest material during close-range searching.

7.3 MATERIALS AND METHODS

7.3.1 Cuticular hydrocarbons on the nest material

Females of *P. triangulum* (N = 12) were caught in front of their nests in beewolf aggregations in Nürnberg and Erlangen, Germany. They were kept in observation cages as described before (Strohm & Linsenmair 1995). Excavated material from the nest mounds was used for the olfactometer bioassay (see below) and then subjected to chemical analysis. The sand was transferred to a 5 ml glass vial and weighed. As an internal standard, 3 µg of octadecane (3 µl of a 1 g/l solution of octadecane in hexane) was added to the sand and the sand was extracted in 4 ml of distilled hexane for 10 min under slight agitation. The hexane was sucked off using a Pasteur pipette and the extracts were reduced in volume by a gentle stream of nitrogen at ambient temperature to a final volume of 50 µl and subjected to GC-MS analysis.

7.3.2 High resolution coupled gas chromatography-mass Spectrometry (GC-MS):

GC-MS analysis was performed with an Agilent 6890N Series gas chromatograph (Agilent Technologies, Böblingen, Germany) coupled to an Agilent 5973 inert mass selective detector. The GC was equipped with a HP-5 fused silica capillary column (J&W, 30 m x 0.32 mm ID; df = 0.25 µm). The temperature program used was 60°C for 1 min, then to 300°C at 5°C/min, held for 10 min. A splitless injector (250°C) was used with helium as the carrier gas at a constant flow of 1 ml/min. Electron ionization mass spectra were recorded at 70 eV.

The substances of the NHCs of beewolf females were identified using mass spectra and retention times from earlier analyses (Strohm et al. 2008). Peak areas were obtained by manual integration, and the amounts of compounds were calculated by comparing peak areas to the internal standard. Some peaks were not separated satisfactorily and were treated as one peak for integration (Table 7.1). In particular, (*Z*)-9- and (*Z*)-7 isomers of alkenes were generally not satisfactorily separated.

7.3.3 Visual and gustatory/olfactory host location:

Females of *H. rutilans* (N = 15) were caught in the vicinity of beewolf nests in a beewolf aggregation in Würzburg, Germany. The bioassays were conducted in a circular plastic arena (height 15 cm, diameter 91 cm) covered with a glass plate with a small hole to introduce the cuckoo wasp. The bottom of the arena was covered with dark green construction paper.

7.3.3.1 Visual host location:

The aim of this experiment was to assess whether cuckoo wasp females are attracted to visual stimuli that mimic a nest mound of their host. The arena was divided in four equally sized sectors. In the middle of each sector we placed one of four stimuli with increasing similarity to original nest mounds: (1) negative control: a paper disc made of dark green construction paper (diameter 12 cm) not standing out against the dark green background, (2) color and size matching a nest mound: a paper disc made of light brown construction paper (diameter

12cm) (3) color, size, and surface structure matching a nest mound: sand (from the nest site but not from nest mounds) spread flatly on a paper disc (diameter 12), (4) color, size, surface structure, and three dimensional shape matching a nest mound: sand piled resembling a beewolf nest mound on a paper disc (diameter 12 cm). Cuckoo wasp females (N = 7) were individually introduced into the arena. The time a cuckoo wasp spent on each object was recorded for 30 min. The absolute positioning of the different visual stimuli was varied systematically to avoid biased results due to possible absolute side preferences of the cuckoo wasps. We predicted that if there was a visual component of host nest location, stimuli 1-4 should cause increasing degrees of attraction

7.3.3.2 Gustatory/olfactory host location:

This bioassay tested the ability of cuckoo wasp females to distinguish sand mounds having been in contact with beewolf females from untreated sand and sand having been in contact with another hymenopteran species. The arena was divided into three equally sized sectors. Each sector was equipped with an open Petri dish (diameter 9 cm) filled with differently treated sand. (1) negative control: untreated sand, (2) CHCs of *Dasypoda hirtipes* (Hymenoptera, Apidae, Melittinae), and (3) CHCs of beewolf females. *D. hirtipes* is a ground nesting solitary bee, which sometimes occurs in the same habitat as beewolves and whose nest mounds look very similar to those of European beewolves (E. Strohm unpubl. data). To obtain sand with CHCs of female *D. hirtipes* and *P. triangulum* an individual of the respective species was kept in a closed sand filled Petri dish for 30 min. Since the individuals tried to escape they vigorously moved through the sand thus contaminating the sand with CHCs. This procedure should result in a variation of the amounts of HCs transferred to the sand how it is likely to occur in nature. Furthermore, due to the similarity in behavior and of the nests the amounts of HCs transferred to the sand by the two species should reflect the natural ratio of HCs encountered by *H. rutilans* females in the field. Cuckoo wasp females (N = 8) were individually introduced into the circular arena and the time spent on each Petri dish was recorded for 30 min. The absolute positioning of the different stimuli was varied systematically to avoid biased results due to possible side preferences of the cuckoo wasps. We predicted that if chemical stimuli play a role for the host nest location, the attractiveness should be lowest for stimulus 1, intermediate for stimulus 2, and highest for stimulus 3.

7.3.3.4 Olfactometer bioassay:

To test the behavioral response of *H. rutilans* females to olfactory stimuli of the nest of beewolf females, we used a four-arm olfactometer after Petterson (1970) and Vet et al. (1983), which was adjusted to the specific requirements of this study. Females of *P. triangulum* (N = 12) were caught in front of their nests in beewolf aggregations in Nürnberg and Erlangen, Germany. Beewolf females were kept in observation cages as described before (Strohm & Linsenmair 1995). Excavated material from the nest mounds was used for the olfactometer bioassay. Two opposing arms were supplied with no odor and served as buffer zones according to Ruther et al. (2007), one arm was supplied with odor from control sand, and one arm was supplied with odor from sand obtained from beewolf nest mounds. The arena of the olfactometer was CNC machined from a single aluminum block (h 350 x l 350 x d 20 mm), resulting in a flat chamber with four lobes and an inner clearance of 15 mm. A glass plate served as top cover of the arena and was tightly attached to the body of the aluminum plate using distilled water. A central hole was drilled in the bottom of the arena to allow the air to drain off the arena. Each arm had a hole at its apex that was connected to an air supply. Holes for in- and outflow of air were obstructed with metal sieves to prevent the wasps from escaping from the olfactometer. For air supply, a small vacuum pump was used (FM1101, EPDM membrane, Fürgut, Germany). The air was purified in a washing flask filled with granulated charcoal (Roth, Germany) and humidified in a washing flask filled with distilled water. Purified and humidified air was split into four tubes leading to four flow meters (Analyt, Germany). Two flowmeters provided air for the odor-free buffer zones through Teflon tubes (diameter 6mm, Roth, Germany). The other two flowmeters provided air for the beewolf nest-odor and the sand control. Plexiglas tubes (30 mm diameter, 12 cm length) were inserted between the flowmeter and the arena and filled with 5 ml of beewolf nest excavations or control sand, respectively. All flowmeters were adjusted to an air-flow of 120 ml/min. To screen the arena from surrounding visual stimuli, it was covered with a steel cylinder (diameter 49 cm, height 50 cm) covered with white paper. The arena was illuminated using two concentric circular fluorescent lamps (21 cm diameter (22 W) and 41 cm diameter (40 W) respectively, Osram, Germany) that were placed on the hood. A screen of white thermoplastic foil was placed underneath the lamps to generate homogenous illumination. A video camera with a keyhole lens was inserted through a small central hole in the foil screen and allowed the observation of the wasp inside the arena over a monitor without disturbing the animals in the arena and without providing any landmark. The temperature in the room was adjusted to 27°C.

Females of *H. rutilans* (N = 16) were caught in the vicinity of beewolf nests in a beewolf aggregation in Nürnberg, Germany. *H. rutilans* females were introduced into the arena through a central hole in the glass cover, which was immediately covered with a cover slip. Each wasp was observed for 15 min and the time it stayed in each quarter of the olfactometer was recorded. The olfactometer was carefully cleaned with ethanol and hexane after each trial. The position of the test odor application was varied systematically to avoid biased results due to possible side preferences of the cuckoo wasps. We predicted that if olfactory cues from beewolf nests are important for cuckoo wasps, they should stay longer in the arm that was supplied with odor from the excavated sand.

7.3.4 Data analysis:

7.3.4.1 Nest hydrocarbons:

For each individual sand extract, the total peak area was standardized to 100%. Because peak areas represent compositional data, they were transformed to logcontrasts (Aitchison 1986; Reyment 1989) before analysis. However, the original transformation procedure makes it necessary to exclude compounds that do not occur in all samples. We, thus, modified the transformation (log10 (relative peak area/(geometric mean of all peak areas)+1)) to avoid undefined values for peaks with an area of zero. However, only very few peaks (7 out of 442) had a zero value and these peaks had no influence on normality.

Females of the European beewolf show a marked dimorphism in the composition of their CHCs (Strohm et al. 2008; see also chapter 8), and we wanted to test the hypothesis that at least the differences of these two types are qualitatively reflected in the sand extracts. Therefore, the transformed relative peak areas were subjected to a principal component analysis (PCA) to reduce the number of describing variables. The extracted PCA factors were subjected to a discriminant analysis (DA) to assess whether the two chemical morphs with pentacosene or heptacosene as the main component could be separated on the basis of the extracts of their nest material. All analyses were calculated by using SPSS 15.0.

To test for a chemical congruency between the substances found in the nest material and on the cuticle of beewolves, we conducted a regression analysis between the proportions of the components (log transformed) present in the nest material samples and in the postpharyngeal gland (PPG) secretion (reduced major axis regression (RMA); Legendre & Legendre 1998) using RMA Software for Reduced Major Axis Regression v.1.17 (A. J.

Bohonak, San Diego University, USA; freely available at http://bio.sdsu.edu/pub/andy/RMA.html). The secretion present in the PPG has been shown to be identical to the CHCs of beewolf females (Strohm et al. 2008). The regression analysis was performed for both chemical morphs separately.

7.3.4.2 Host location of *H. rutilans*:

Data on the time of *H. rutilans* females spent on different visual and gustatory/olfactory stimuli in the circular arena were compared using exact Friedman ANOVA followed by Wilcoxon exact tests. Data on residence time spent in the test and the control arm in the olfactometer bioassay were normally distributed according to a Kolomogorow-Smirnow test and were compared with a paired t-test (SPSS 15.0).

7.4 RESULTS

7.4.1 CHCs in the nest material of *P. triangulum* females

In the extracts of nest material of beewolf females, we found a total of 34 substances (Table 7.1). We found a total amount of 3.61 ± 2.05 µg (X ± SD) hydrocarbons per gram excavated nest material (range: 1.36 µg to 8.21 µg). As in the CHC composition, we found a marked dimorphism in the composition of the NHCs with either pentacosene (C_{25}-type) or heptacosene (C_{27}-type) as the most abundant substances (Fig. 7.1, Table 7.1). To test whether these differences are significant we analyzed the chemical variability of the sand extracts using multivariate data analysis. The PCA revealed 5 principal components, which explained 97.4 % of the variance. The DA on these principal components significantly differentiated extracts from C_{25}-type and C_{27}-type females (Wilk's $\lambda = 0.011$, $\chi^2_5 = 38.7$, $N = 13$, $P < 0.001$) and revealed one discriminant function. Discriminant function 1 explained 100% of the variance.

To test for a chemical congruency between the substances found in the nest material and on the cuticle of beewolves, a total of 20 peaks (log transformed) were subjected to the regression analysis. Several substances had to be combined for the analysis, because peaks

were not always well separated. The relative amounts of substances in the PPG showed a strong linear correlation with the corresponding substances in the nest material (C_{25}-type: $y = 0.7239x + 0.5507$, $r^2 = 0.501$; C_{27}-type: $y = 0.7565x + 0.5247$, $r^2 = 0.542$).

Table 7.1: Chemical composition of the nest hydrocarbons (NHCs) of beewolf females, which had either pentacosene (C_{25}-type, N = 8) or heptacosene (C_{27}-type, N = 5) as the main component. Peak numbers correspond to numbers given in Fig. 7.1. Given are the means and standard deviations (SD) for the percentages of the different compounds in both types.

		C_{25}-type		*C_{27}-type*	
No.	***Compound***	***Mean***	***SD***	***Mean***	***SD***
1	*C21*	*0.24*	*0.09*	*0.21*	*0.07*
2	*C22*	*0.33*	*0.15*	*0.27*	*0.08*
3	*C23:1*	*1.18*	*0.29*	*0.91*	*0.23*
4	*C23:1*	*0.21*	*0.07*	*0.14*	*0.05*
5	*C23*	*15.57*	*3.70*	*13.75*	*3.48*
6	*9,11meC23*	*0.16*	*0.06*	*0.08*	*0.05*
7	*5meC23*	*0.08*	*0.07*	*0.07*	*0.07*
8	*C24:1*	*1.00*	*0.17*	*0.15*	*0.02*
9	*C24:1*	*0.16*	*0.19*	*0.11*	*0.09*
10	*C24*	*0.97*	*0.46*	*1.07*	*0.31*
11	*C25:1*	*39.60*	*5.10*	*4.72*	*0.80*
12	*C25*	*14.95*	*3.41*	*20.17*	*3.70*
13	*11,13meC25*	*0.16*	*0.04*	*0.14*	*0.03*
14	*C26:1*	*0.31*	*0.06*	*1.22*	*0.24*
15	*C26*	*1.09*	*0.89*	*1.23*	*0.47*
16	*C25on*	*0.68*	*0.58*	*0.53*	*0.28*
17	*C27:1 (2x)*	*2.84*	*0.55*	*32.64*	*5.89*
18	*C27*	*6.82*	*1.55*	*7.88*	*1.93*
19	*11,13meC27*	*0.19*	*0.06*	*0.16*	*0.03*
20	*C28*	*0.91*	*0.74*	*1.04*	*0.40*
21	*C29:1 (2x)*	*0.49*	*0.15*	*2.09*	*0.25*
22	*C29*	*5.60*	*0.94*	*6.39*	*1.21*
23	*11,13,15meC29*	*0.14*	*0.04*	*0.10*	*0.02*
24	*C30*	*0.56*	*0.40*	*0.60*	*0.22*
25	*C31:2*	*0.11*	*0.07*	*0.13*	*0.10*
26	*C31:2*	*0.11*	*0.06*	*0.10*	*0.07*
27	*C31:1*	*0.74*	*0.57*	*0.58*	*0.12*
28	*C31:1*	*0.48*	*0.36*	*0.31*	*0.10*
29	*C31*	*2.35*	*0.31*	*2.03*	*0.59*
30	*13,15meC31*	*0.10*	*0.07*	*0.07*	*0.02*
31	*C32*	*0.13*	*0.09*	*0.12*	*0.06*
32	*C33:2*	*0.23*	*0.17*	*0.13*	*0.04*
33	*C33:1*	*1.30*	*1.17*	*0.69*	*0.22*
34	*C33*	*0.22*	*0.06*	*0.18*	*0.08*

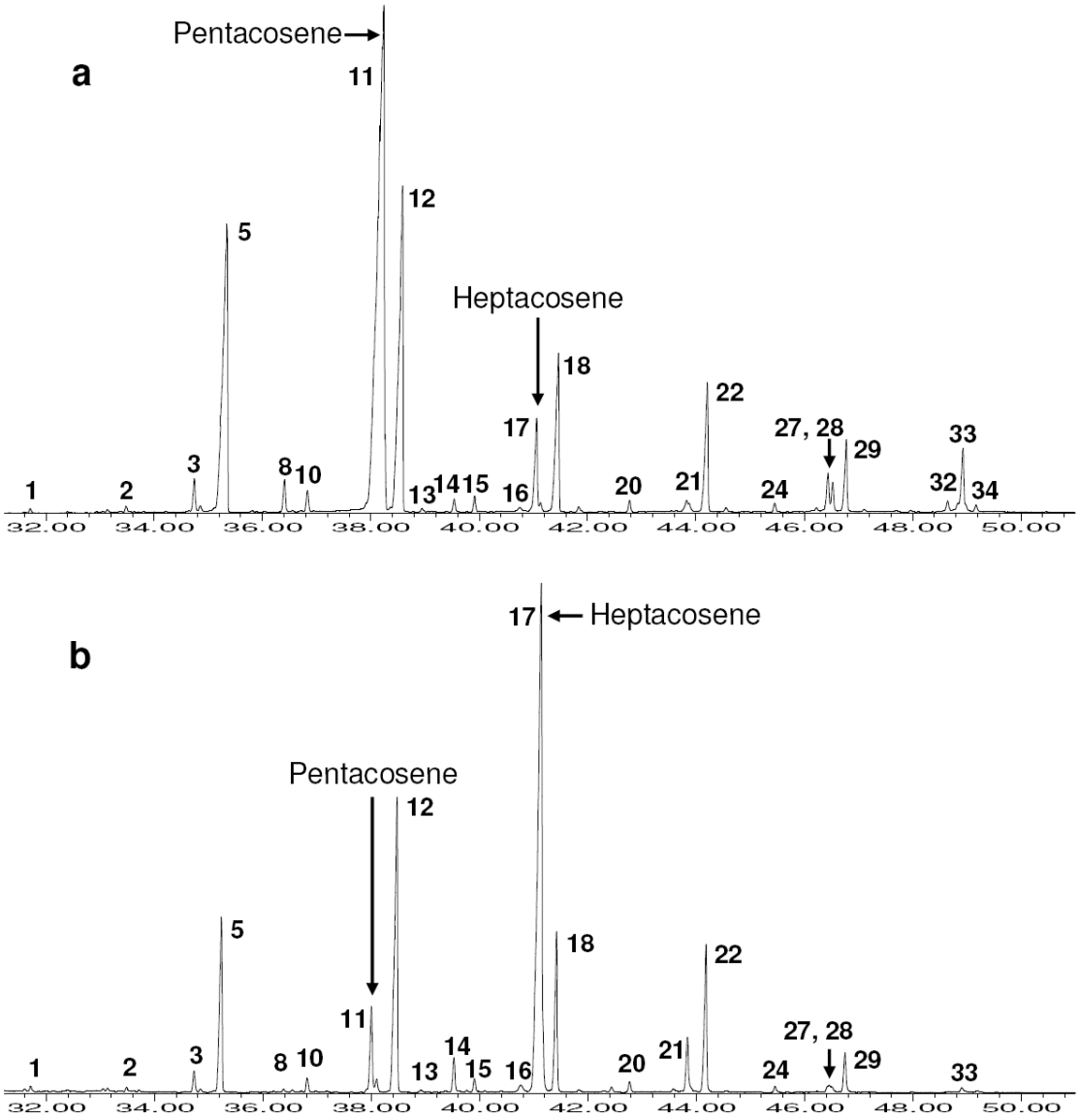

Fig. 7.1: Total ion chromatogram of NHCs of beewolf females having either pentacosene (C_{25}-type, a) or heptacosene (C_{27}-type, b) as the predominant peak. Peak numbers correspond to numbers given in Table 7.1.

7.4.2 Host location of *Hedychrum rutilans* by visual stimuli

H. rutilans females stayed on the artificial sand mounds longer than on the light brown paper discs and the negative control (Fig. 7.2, Friedman exact test: $\chi^2_{\ 3} = 19.5$, N = 7, P < 0.001, Wilcoxon exact tests, see Fig. 7.2 for the results of the tests). This suggests that not only a high contrast with respect to the background but also the actual shape of the stimulus is essential for the attraction of the cuckoo wasps. Additionally, the material texture of the

stimulus seems to be important, since cuckoo wasps spent more time searching on the flat sand than on paper disks or on the negative control area. Although the mean time spent on the piled sand was considerably higher than for the flat wand this comparison was not significant (P = 0.297).

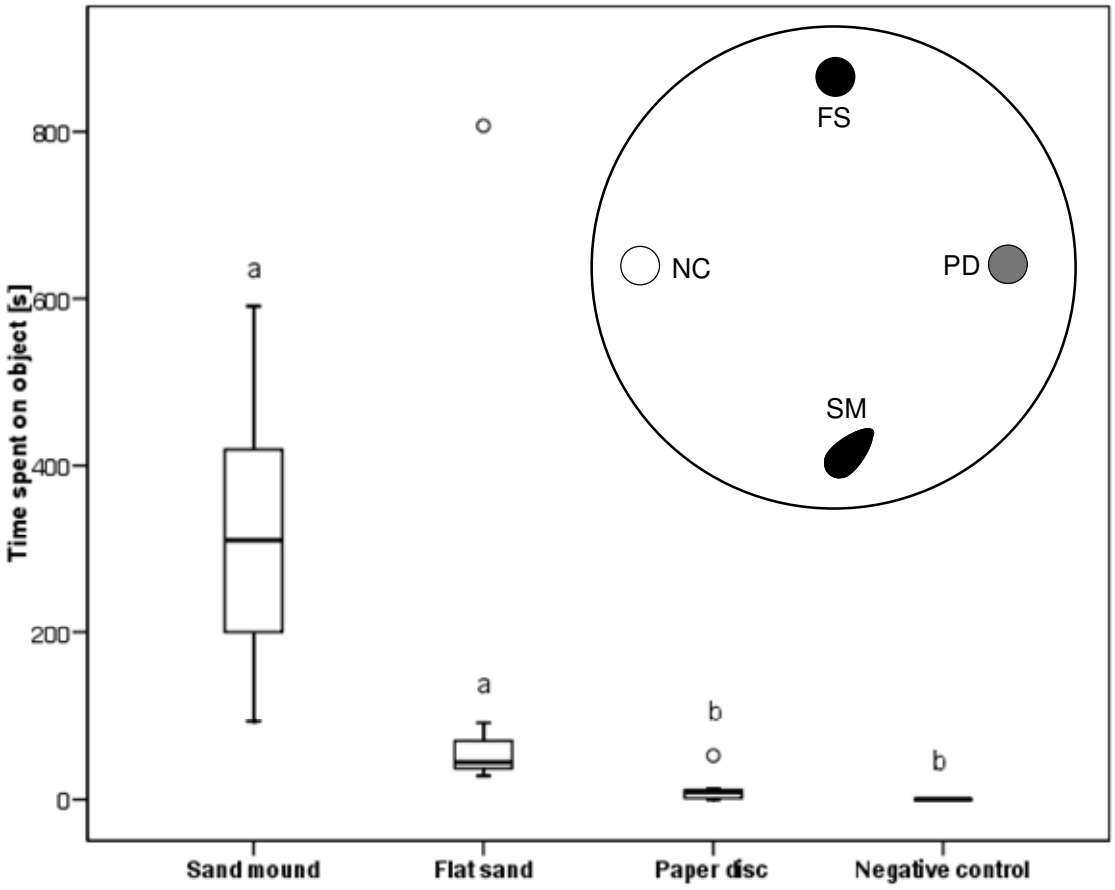

Fig. 7.2: Visual host location: time spent on different visual stimuli of the arena. Bold lines represent medians, boxes comprise the interquartile range, bars indicate minimum and maximum values, and circles indicate outliers (Friedman exact test: $\chi^2_3 = 19.5$, N = 7, P < 0.001). Different letters above the boxes indicate significant differences between stimuli (Wilcoxon exact tests: P < 0.05). The figure inset shows the schematics of the arena with four different visual stimuli (SM: sand mound, FS: flat sand, PD: paper disc; NC: negative control).

7.4.3 Host location of *Hedychrum rutilans* by gustatory and/or olfactory stimuli

Cuckoo wasp females spent significantly more time on the sand in which a beewolf female had been allowed to dig as compared to both a negative control (Fig. 7.3, Friedman exact test: $\chi^2_2 = 10.13$, N = 8, P < 0.01, Wilcoxon exact tests, see Fig. 7.3 for the results of the tests) and the sand contaminated with CHCs of *Dasypoda* bees.

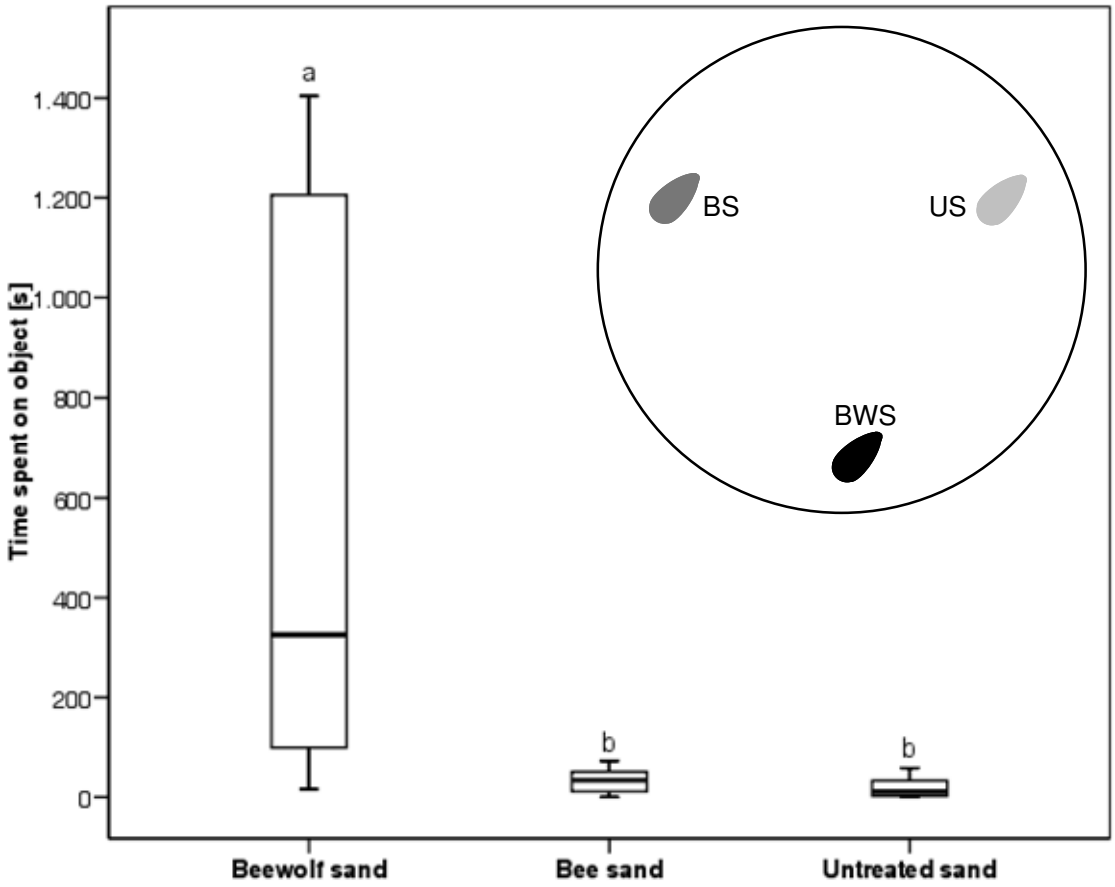

Fig. 7.3: Gustatory/olfactory host location: time spent on Petri dishes filled with differently treated sand. (Friedmann exact test: $\chi^2_2 = 10.13$, N = 8, P < 0.01). Different letters above boxes indicate significant differences between groups (Wilcoxon exact tests: P < 0.05). The figure inset shows the schematics of the arena with three different gustatory/olfactory stimuli (BWS: beewolf sand, BS: bee sand, US: untreated sand).

7.4.4 Host location of *Hedychrum rutilans* by olfactory stimuli

Females of *H. rutilans* stayed significantly longer in the test area of the olfactometer (X ± SD = 387 ± 306 s) as compared to the control arm of the olfactometer (X ± SD = 155 ± 170 s; paired t-test: $t_{16} = 2.242$, P < 0.05; Fig. 7.4). Thus, the cuckoo wasps were attracted to the nest odor of their beewolf hosts. We did not find a correlation between the amount of hydrocarbons present in the nest material and the time spent in the test arm of the olfactometer (Pearson r = -0.258, N = 16, P = 0.334).

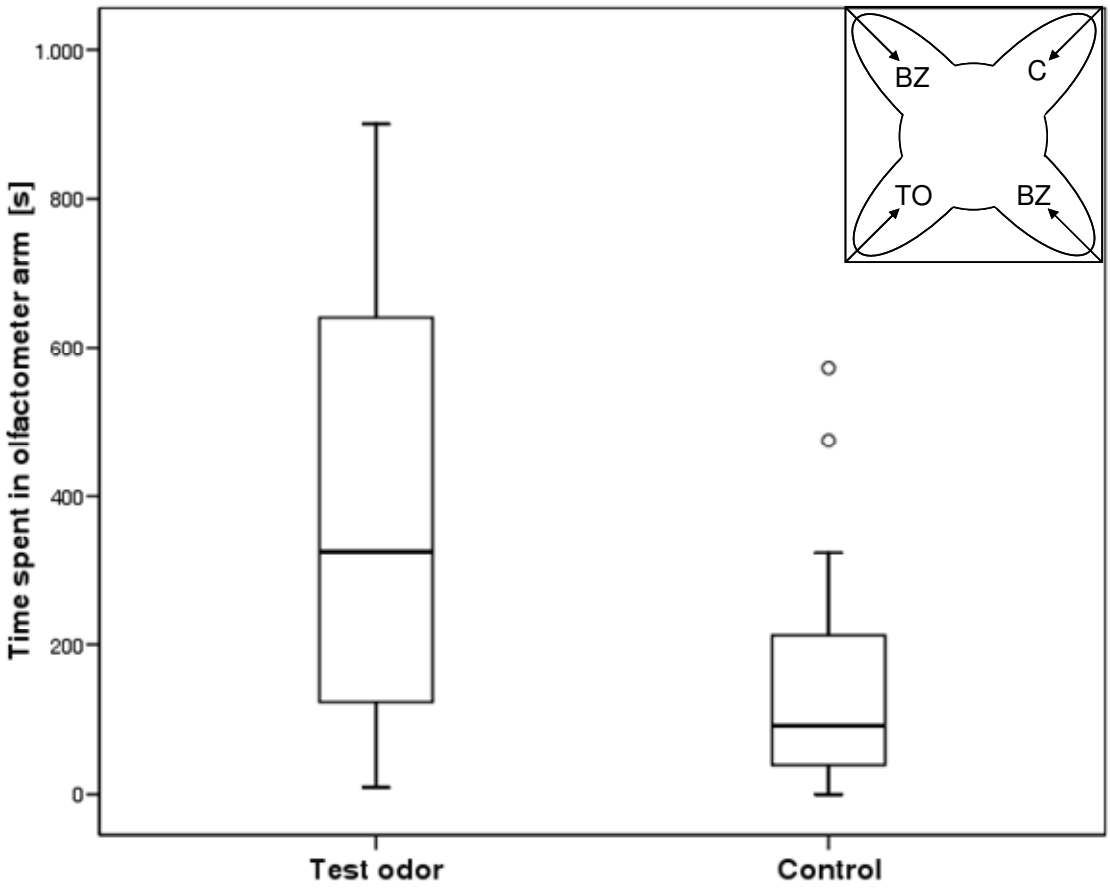

Fig. 7.4: Olfactory host location: time that *H. rutilans* females spent in test odor and control section of a four-arm olfactometer. Test odor fields obtained air that had passed over 5 ml of nest mound material of individual beewolf nests. The air in the control fields had passed over 5 ml of untreated sand. The time spent in the two field is significantly different (paired t-test, T = 2.24, df = 15, P < 0.05). The figure inset shows the schematics of the four-arm olfactometer with the air running into the four arms of the arena (TO: test odor, C: control, BZ: buffer zone).

7.5 DISCUSSION

Our results clearly show that excavated nest material of female European beewolves contains surprisingly large amounts of CHCs, thus representing NHCs. To date, NHCs had only been described for social paper wasps (Espelie & Hermann 1990; Lorenzi & Caprio 2000; Singer et al. 1992; Singer & Espelie 1996; Sumana et al. 2005). Thus, the data presented here constitute the first description of NHCs in a ground-nesting insect and the first report of NHCs in a solitary species of the Hymenoptera.

The NHCs of the European beewolf qualitatively closely resemble the CHC composition of the beewolf females (Strohm et al. 2008). Based on the composition of the NHCs, it was clearly

possible to discriminate between the two chemomorphs found in *P. triangulum*. There are two supposable ways how the CHCs could be transferred to the nest material: First, substances from the beewolves' cuticle could be transferred from the legs and the abdomen to the nest walls during digging and processing of sand in the nest. Second, the compounds might be transferred from prey bees that are treated by the beewolves with a secretion from the PPG, which is identical to the CHCs (Strohm et al. 2008). Beewolf females apply huge amounts of the secretion onto the bees by extensive licking. This secretion has the function to delay fungus growth on the larval provisions in the humid environment of the underground nest (Herzner & Strohm 2007). During the excavation of the side burrow, the treated bees are frequently moved around in the nest (Simon-Thomas & Veenendaal 1978). Therefore, the secretion might be easily transferred to the nest wall material and the loose sand that is transported to the nest entrance and ejected. Whether transfer of chemicals from the females' bodies or from the treated bees is more important awaits further studies on species that do not treat their prey. Assuming that prey treatment is essential, an accumulation of NHCs in the nest material might be unique to beewolves.

We propose that the presence of the NHCs of the beewolf nest is the result of strictly passive and unintended transfer from the beewolf cuticle and treated bees to the nest material. Concordantly, no particular behavior can be observed that might be involved in active nest-marking (Kroiss and Strohm unpubl. data). Nevertheless, beewolf females might use the presence of these olfactory cues themselves especially in the context of close-range nest-finding (see Wilms & Eltz 2007). While beewolves use optical cues for the remote localization of their nests (Tinbergen 1932), Tinbergen's reports provide some evidence for an additional use of olfactory cues for the close range nest identification. So far, little is known about the passive transfer of CHCs to nest (entrance) material and their role as a nest recognition cue (see Grasso et al. 2005 for nest marking with faeces containing colony-specific hydrocarbons in harvesting ants; but see Jandt et al. 2005; Steinmetz et al. 2002). In contrast, there are several reports about active nest marking with secretions from various glands and the role of olfactory cues for nest finding and nest recognition in solitary (Guedot et al. 2006; but see Inouye 2000 for the absence of olfactory nest-finding in the a ground-nesting bee) and social Hymenoptera (Cammaerts & Cammaerts 1998; Cammaerts & Cammaerts 2001; Hölldobler & Wilson 1990).

For the specialized cuckoo wasp *H. rutilans* the nests of the European beewolf seem to provide both visual and chemical cues. Our bioassays show that cuckoo wasps are visually attracted by the characteristic shape and texture of the beewolf nest mound. Apart from chemical cues, visual stimuli have been frequently shown to be involved in host location in parasitic insects (Keasar et al. 2000; Morehead & Feener 2000).

The bioassays that tested for possible chemical cues from beewolf nests suggest that female *H. rutilans* perceive the presence of NHCs in the nest mounds of beewolves and use it for close-range host localization and identification. The results of the olfactometer experiment show that cuckoo wasps even respond to olfactory cues of nest material. This olfactory detection of the NHCs by the cuckoo wasps is surprising since the compounds in question have are rather long chained and consequently have a low but not zero volatility (see e.g. Schmitt et al. 2007). This suggests that cuckoo wasps are endowed with a very high sensory sensitivity for these compounds.

It is known that parasitoids use either microhabitat derived cues or host derived cues for host location and assessment of host quality (Godfray 1994). Generalist parasitoids tend to use microhabitat derived cues whereas specialized parasitoids use host derived cues (Cortesero et al. 1997; De Moraes & Mescher 2005; Schönrogge et al. 2008; Vet & Dicke 1992). Our findings in the specialized cuckoo wasp *H. rutilans* confirm the pattern that specialists preferentially make use of host derived substances. Host derived cues might be either substances purposely emitted for intra- or interspecific communications (Mizutani 2006; sex- or aggregation pheromones, defensive secretions; Geiselhardt et al. 2006; Godfray 1994), substances located on the cuticle (Colazza et al. 2007; Howard & Blomquist 1982; Jones et al. 1973; Jones et al. 1971; Lewis et al. 1976; Morehead & Feener 2000; Muratori et al. 2006; Singer 1998; Vinson et al. 1975) or other substances associated with the host (e.g. Ruther & Steidle 2000). Since *H. rutilans* cuckoo wasps are able to detect and identify beewolf nests based on NHCs, selection should act on the beewolves to reduce the amount of these chemicals in the nest mound material. However, this is probably opposed by a strong selection pressure to apply large amounts of the PPG secretion onto the bee prey to avoid molding in the brood cell, which constitutes a major mortality risk for the developing larva (Herzner et al. 2007; Herzner & Strohm 2007; Strohm & Linsenmair 2001). However, beewolf females might reduce the olfactory conspicuousness of their nests by nesting in steep sandy slopes as opposed to flat ground. In that case, ejected nest material will not pile up in front of the nest and form the characteristic nest mound, but will fall to the base of the slope and will be dispersed away from the nest. In fact, beewolf females prefer to nest in steep slopes if available and might thereby reduce the risk of parasitism.

In conclusion, our results show that nest mounds of European beewolves contain large quantities of hydrocarbons that resemble the composition of the CHCs of beewolf females. Additionally, we found clear evidence that the NHCs serve as a kairomone for the specialized cuckoo wasp *H. rutilans*. In combination with visual cues these NHCs might facilitate host localization and identification. We hypothesize that the NHCs might play a role for optimal host choice of the cuckoo wasps.

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CHAPTER 8

A CUCKOO IN WOLVES' CLOTHING? CHEMICAL MIMICRY IN A SPECIALIZED CUCKOO WASP OF THE EUROPEAN BEEWOLF.

Frontiers in Zoology: 5:2

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8.1 SUMMARY

Background: Host-parasite interactions are among the most important biotic relationships. Host species should evolve mechanisms to detect their enemies and employ appropriate counterstrategies. Parasites, in turn, should evolve mechanisms to evade detection and thus maximize their success. Females of the European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae) hunt exclusively honeybee workers as food for their progeny. The brood cells containing the paralyzed bees are severely threatened by a highly specialized cuckoo wasp (*Hedychrum rutilans*, Hymenoptera, Chrysididae). Female cuckoo wasps enter beewolf nests to oviposit on paralyzed bees that are temporarily couched in the nest burrow. The cuckoo wasp larva kills the beewolf larva and feeds on it and the bees. Here, we investigated whether *H. rutilans* evades detection by its host. Since chemical senses are most important in the dark nest, we hypothesized that the cuckoo wasp might employ chemical camouflage.

Results: Field observations suggest that cuckoo wasps are attacked by beewolves in front of their nest, most probably after being recognized visually. In contrast, beewolves seem not to detect signs of the presence of these parasitoids neither when these had visited the nest nor when directly encountered in the dark nest burrow. In a recognition bioassay in observation cages, beewolf females responded significantly less frequently to filter paper discs treated with a cuticular extract from *H. rutilans* females, than to filter paper discs treated with an extract from another cuckoo wasp species (*Chrysis viridula*). The behavior to paper discs treated with a cuticular extract from *H. rutilans* females did not differ significantly from the behavior towards filter paper discs treated with the solvent only. We hypothesized that cuckoo wasps either mimic the chemistry of their beewolf host or their host's prey. We tested this hypothesis using GC-MS analyses of the cuticles of male and female beewolves, cuckoo wasps, and honeybee workers. Cuticle extracts of *Hedychrum nobile* (Hymenoptera: Chrysididae) and *Cerceris arenaria* (Hymenoptera: Crabronidae) were used as outgroups. There was little congruence with regard to cuticular compounds between *H. rutilans* females and honeybees as well as females of *C. arenaria* and *H. nobile*. However, there was a considerable similarity between beewolf females and *H. rutilans* females. Beewolf females show a striking dimorphism regarding their cuticular hydrocarbons with one morph having (*Z*)-9-C25:1 and the other morph having (*Z*)-9-C27:1 as the major component. *H. rutilans* females were more similar to the morph having (*Z*)-9-C27:1 as the main component.

Conclusions: We conclude that *H. rutilans* females closely mimic the composition of cuticular compounds of their host species *P. triangulum*. The occurrence of isomeric forms of certain compounds on the cuticles of the cuckoo wasps but their absence on beewolf females suggests that cuckoo wasps synthesize the cuticular compounds rather than sequester them from their host. Thus, the behavioral data and the chemical analysis provide evidence that a specialized cuckoo wasp exhibits chemical mimicry of the odor of its host. This probably allows the cuckoo wasp to enter the nest with a reduced risk of being detected by olfaction and without leaving traitorous chemical traces.

8.2 BACKGROUND

The interaction between hosts and parasites or parasitoids is one of the most important forces driving evolutionary and ecological processes (e.g. Godfray 1994). In order to reduce the impact of parasitoids, host species may evolve mechanisms to detect their enemies and employ adequate counterstrategies (e.g. Quicke 1997; Rosenheim 1988; Strohm et al. 2001;

e.g. Tengo & Bergstrom 1977). Parasitoids, in turn, are selected to evolve mechanisms that reduce the probability of being detected by their hosts to circumvent such countermeasures. This sets the stage for repeated cycles of adaptations and counteradaptations ("evolutionary arms race" (see e.g. Brandt et al. 2005; Davies et al. 1989; Dawkins & Krebs 1979; Foitzik et al. 2003; Lyon & Eadie 2004) between hosts and parasites, especially if the parasitoid is highly specialized on a single host species and has a large impact on host fitness (Sick et al. 1994; Spencer 1998; Tengo & Bergstrom 1977).

Progeny of brood caring bees and wasps are particularly susceptible to parasitism (Evans & O'Neill 1988; Jacob-Remacle 1986; Wilson 1971). Females of these species store large amounts of valuable nutrients as larval provisions in brood cells. These valuable resources attract a variety of brood parasites, either cleptoparasites that reduce the amount of resources available to the host's progeny or parasitoids that obligatorily kill the host larvae. Mostly, females of these parasitic species have to enter the nest or the brood cell to deposit eggs or larvae. Thus, the traces that are left by female brood parasites might be detected by the host. As a result, the hosts might abandon the nests or remove or destroy eggs of brood parasites (Kimsey & Bohart 1990; Rosenheim 1988). Brood parasites might also be encountered in the nest by the host and might be driven away, injured, or even killed (E. Strohm, unpubl. observations). Since insects heavily rely on their chemical senses for any kind of recognition or localization process (Godfray 1994; Herzner et al. 2005; Jackson & Morgan 1993), concealment of a brood parasite's actual or previous presence will require chemical camouflage (compounds sequestered from the host or the host's nest) or chemical mimicry (compounds synthesized by the mimic, definitions sensu (Dettner & Liepert 1994)). In this study, we investigated the interaction between a hunting wasp, the European beewolf, *Philanthus triangulum* (Hymenoptera, Crabronidae) and its highly specialized brood parasitoid *Hedychrum rutilans* (Hymenoptera, Chrysididae). We investigated the following questions: Are cuckoo wasps (*H. rutilans*) detected and recognized by beewolf females at all? Is there a difference in host response towards the cuckoos outside and inside the nest and do the cuticular hydrocarbons play a role for the detection of the cuckoo wasps inside the nest? Is the chemical composition of the cuticular hydrocarbons of *H. rutilans* females similar to their host or to their host's prey?

Females of the European beewolf hunt honeybee workers (Hymenoptera, Apidae) as food for their progeny. Several paralyzed bees are temporarily couched in the main burrow (up to 1m long) of the underground nest (see (Strohm 1995) for details on nest architecture). Eventually, the female closes the nest entrance, excavates a side burrow and a terminal brood cell, brings in one to six paralyzed bees, and oviposits on one of the bees (Strohm &

Linsenmair 1999). Thereupon, she carefully closes the side burrow and subsequently has no contact to her progeny.

The cuckoo wasp, *H. rutilans*, is a specialized brood parasitoid of the genus *Philanthus* (Kimsey & Bohart 1990; Kunz 1994). However, since in Central Europe only one member of the genus, *P. triangulum*, is fairly abundant, *H. rutilans* is effectively monospecific in this region. This considerable degree of specialization is expressed by the unique oviposition strategy of *H. rutilans*. Most chrysidid wasps oviposit into the brood cell of their hosts at a defined stage of the provisioning cycle or after the brood cell has been finally closed (Kunz 1994). In beewolves, however, the brood cell is excavated only after the female has brought in the bees and the nest entrance has been closed. Thus, the nest is blocked up and the female is attendant until the brood cell is finally closed. This leaves little opportunity for a cuckoo wasp to deposit an egg in the brood cell. As a consequence, *H. rutilans* females pursue two alternative strategies. Either they rapidly pounce and oviposit on a paralyzed bee when the female alights with its prey and enters the burrow (Veenendaal 1987, E. Strohm unpubl. observation), or *H. rutilans* females wait in front of the nest until the host female leaves to forage and then enter the burrow and oviposit on the paralyzed bees that are temporarily couched there (Kunz 1994). Thus, *H. rutilans* use the paralyzed bees as a Trojan horse to bring the egg into the brood cell. The latter seems to be the much more frequent mechanism. The mobile larva of *H. rutilans* climbs onto the beewolf larva, kills it, and feeds on the host larva and the bees. Thus, infestation by H. rutilans inevitably leads to a fitness reduction of the host. *H. rutilans* is considered to be the most important brood parasite of *P. triangulum*. The rate of parasitism varies between 3% and more than 30% (Simon-Thomas & Simon-Thomas 1972; Strohm et al. 2001; Strohm & Linsenmair 2000; Strohm et al. 2002, E. Strohm, unpubl. data). *H. rutilans* might even drive local aggregations of *P. triangulum* to extinction (Simon-Thomas & Simon-Thomas 1972).

In both oviposition strategies, detection of the cuckoo wasp female by the beewolf female might decrease the cuckoo wasp's success. First, when encountered in the nest, cuckoo wasps might be carried to the nest entrance by beewolf females and thrown out (Olberg 1953). Mostly, cuckoo wasps are not severely harmed due to the solidity and strong sculpturing of their cuticle and their ability to adopt a rolled-up defensive posture that protects the most vulnerable parts of the body (legs, mouthparts, antennae; see Gauld & Bolton 1996; Kunz 1994). Nevertheless, the wings are rather unprotected and might be injured by a beewolf female. Second, if beewolves females detect signs of the presence of cuckoo wasps they might remove bees from the nest that have possibly been parasitized (Simon-Thomas & Simon-Thomas 1972). Thus, a cuckoo wasp should avoid detection to minimize wastage of time and investment. This means that cuckoo wasp females should avoid detection when they are encountered by a host female in the nest. However, it would probably be even more

important for the cuckoo wasps not to leave any detectable traces of their presence when they had entered the nest and oviposited on a paralyzed honeybee.

In order to assess whether beewolf females respond to the presence of *H. rutilans* females at all we observed the behavioral interactions outside of the nest. To test whether *H. rutilans* females employ chemical camouflage inside the nest we conducted to sets of behavioral experiments. First, in observation cages we recorded the interaction of the cuckoo wasps with beewolf females inside the nest burrow. Second, we conducted a recognition bioassay by assessing the response of beewolf females towards filter discs treated with different extracts: solvent only, cuticular extracts of another chrysidid, *Chrysis viridula*, and cuticular extracts of *H. rutilans*. We predicted that beewolf females should ignore the discs treated with solvent only (negative control), they should respond to the discs treated with *C. viridula* extract (positive control) and they should not (or only weakly) respond to *H. rutilans* extracts.

There are two evolutionary options for *H. rutilans* females to avoid olfactory detection by beewolves. First, cuckoo wasps could mimic the odor of the honeybees that are temporarily couched in the main burrow. Second, *H. rutilans* females might mimic their beewolf host. We consider the imitation of the cuticular compounds of the beewolf host the better alternative, since the host's hydrocarbon profile can be found all over the nest walls due to the digging activity (Kroiss and Strohm unpubl. data) and also on the honeybees. This is because in order to prevent the paralyzed bees from molding they are treated by the beewolf females with a secretion from the postpharyngeal gland that is identical to the beewolves' cuticular hydrocarbons (Herzner et al. 2007; Herzner & Strohm 2007; Herzner & Strohm accepted).

To assess both alternatives, we analyzed the composition of cuticular compounds of beewolf females, cuckoo wasps, and honeybees. Furthermore, we included beewolf males as the a priori most similar group to beewolf females and, thus, a crucial comparison for assessment of this hypothesis. To control for the possibility that chrysidids and crabronids have similar patterns of cuticular hydrocarbons by chance we also analyzed closely related species: the chrysidid wasp *Hedychrum nobile* and its crabronid host *Cerceris arenaria* (subfamily Philanthinae, a weevil hunting wasp). A reasonable null hypothesis for the resemblance among the species under study might be based on their phylogenetic relationship. Crabronids and apids are closely related and constitute the superfamily Apoidea, whereas chrysidids branch off very early (e.g. Brothers 1999; Ronquist 1999). Thus, the null hypothesis would predict that the cuticular profiles of beewolf females should be most similar to conspecifics males and Cerceris females, fairly similar to honeybees, and least similar to cuckoo wasps. Accordingly from a phylogenetic point of view, the chemical profiles of the two congeneric chrysidid species should be most similar to each other.

8.3 RESULTS

8.3.1 Are cuckoo wasps detected by beewolf females outside the nest?

Cuckoo wasps flew over the beewolf nesting site and selectively landed on the mounds of beewolf nests. During the 54 hours of observation we recorded 1024 landings of *H. rutilans* on beewolf nest mounds. In 259 cases (25.3%), cuckoo wasps flew off after ≤ 4 seconds. In 765 cases (74.7%) they remained on or in the vicinity of the nest mound for ≥ 4 seconds, the duration of these stays was 74 ± 250 s (median = 11 s). During these stays the cuckoo wasps moved on the nest mound, vigorously antennating the surface. During prolonged stays at the nest, cuckoo wasps often moved to shaded areas and sometimes even placed themselves under some nearby structures like leaves. In 37 of the 765 cases the entrance was open and cuckoo wasps entered the nest for 13 - 270 s (mean: 118 ± 133 s, median: 60 s). In the remaining 728 cases the entrance was closed. Nevertheless, in 41 of these cases cuckoo wasps tried to dig through the closure; in 29 cases they abandoned digging after some time. In the 12 remaining cases they dug through the nest closure and stayed in the nest for 14 to 1263 s (mean: 384 ± 421 s, median 213 s). During the observation time we observed 89 beewolf females returning with a paralyzed honeybee and entering their nests. In four of these cases we observed attempts of cuckoo wasp females to attach to a honeybee that was carried by a beewolf female while entering the nest with its prey. In all four cases, the female detected the parasitoid and drove it away. In another 11 cases (of the 89) cuckoo wasps were driven away by homing beewolf females although they did not approach the prey laden female to oviposit. In one of these cases the beewolf female grasped the cuckoo wasp with her mandibles. Sometimes (46 cases of 765 cases), cuckoo wasps were driven away from a nest mound by the approach of another cuckoo wasps. Although in one of the 89 cases the cuckoo wasp was in the nest when the beewolf female returned with a bee, it was not thrown out of the nest.

8.3.2 Are cuckoo wasps detected inside the nest?

8.3.2.1 Experiment I

In observation cages in the laboratory, *H. rutilans* females (N = 7) were observed to enter beewolf nests (N = 6 beewolf nests) and oviposit on the couched bees (N = 4). Although in

five cases the beewolf female entered the nest while a cuckoo wasp was present and came close to (less than 2 cm, N = 5) or even passed (N = 3) the cuckoo wasp in the burrow, the host female did not show any signs of detection of the brood parasitoid or disturbance. Notably, the cuckoo wasp either ran to a distant part of the nest when a beewolf female approached or it remained motionless at the periphery of the nest burrow until the female had passed.

8.3.2.2 Experiment II

In the recognition bioassay (Fig. 8.1), beewolf females always responded to the positive control (*C. viridula* extract), they never responded to the negative control (solvent only), and they only rarely responded to the test discs (*H. rutilans* extract). The difference in response frequency between *H. rutilans* and *C. viridula* extracts was significant (Fisher's exact test: P = 0.0101, Fig. 8.1) whereas there was no statistical difference between *H. rutilans* extracts and the negative control (Fisher's exact test: P = 1, Fig. 8.1). This result shows that the chemical signal consisting of cuticular extracts of *H. rutilans* elicits much weaker behavioral responses in beewolf females than extracts of a closely related chrysidid species. Thus, we hypothesized that *H. rutilans* females are chemically camouflaged.

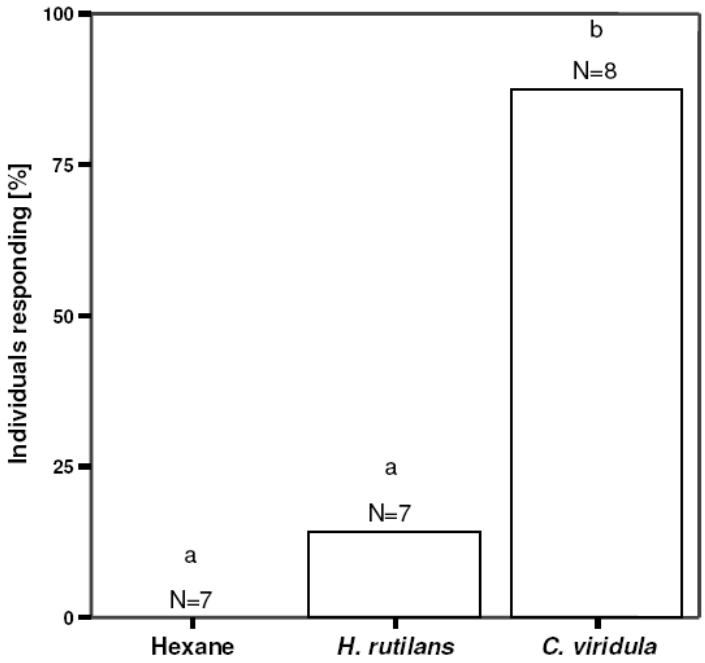

Fig. 8.1: Recognition bioassay: Percentage of beewolf females showing a response towards filter paper discs treated with hexane (negative control, left), cuticular extract of *H. rutilans* females (middle), and cuticular extract of *C. viridula* females (right). Different letters above the bars indicate significant differences between groups (Fisher's exact test: P < 0.05).

8.3.2.3 Are cuckoo wasps chemically cloaked?

The GC-MS analyses revealed alkanes, alkenes, and mono- and dimethylalkanes as the predominant hydrocarbons in all species. We found between 12 and 34 substances on the cuticles of the five species (Table 8.1). *H. rutilans* females shared 10 compounds with beewolf females, 9 with beewolf males, 13 with *H. nobile*, 11 with honeybees, and 14 with *C. arenaria* females.

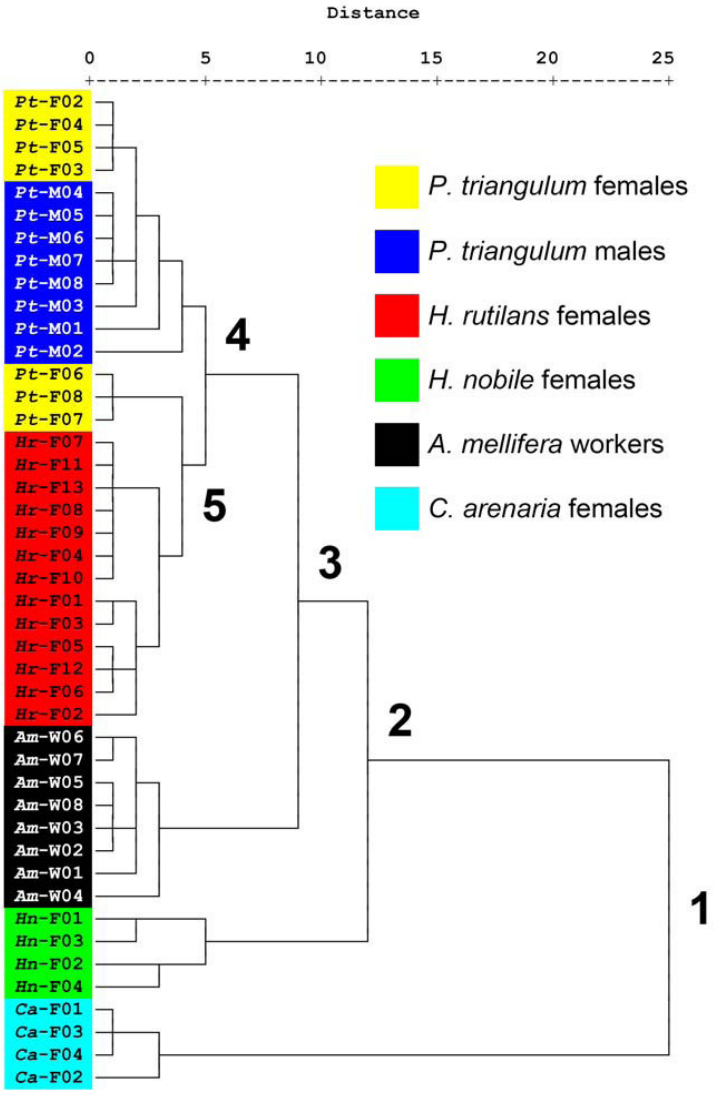

Fig. 8.2: Dendrogram based on the cluster analysis of the cuticular compounds. Included are individual *P. triangulum* females (= *Pt*-F), *P. triangulum* males (= *Pt*-M), *H. rutilans* females (= *Hr*-F), *A. mellifera* workers (= *Am*-W), *C. arenaria* females (= *Ca*-F), and *H. nobile* females (= *Hn*-F). Numbers after the species label indicate the different individuals. Numbers in the dendrogram indicate the first 5 bifurcations (see text).

The cuticles of honeybees contained a varying proportion of saturated and unsaturated hydrocarbons with a chain lengths ranging from C25 to C33. The profile of beewolf females is characterized by a very high proportion of unsaturated hydrocarbons with individuals showing either (*Z*)-9-C25:1 or (*Z*)-9-C27:1 as the main peak (for the source and possible function of this chemical dimorphism see (Herzner et al. 2007; Herzner & Strohm 2007; Herzner & Strohm in press; Strohm et al. 2007; Strohm et al. 2008; Strohm et al. in prep). Cuckoo wasps also show relatively large amounts of the unsaturated C25:1 and C27:1. In contrast to beewolves where individuals had only large proportions of one of these unsaturated compounds, *H. rutilans* females showed fairly large proportions of both. Furthermore, cuckoo wasps had similar proportions of the (*Z*)-7- and the (*Z*)-9-isomers of both substances (Table 8.1).

A cluster analysis based on the proportions of cuticular hydrocarbons as revealed by GC-MS (Fig. 8.2) clearly separated *C. arenaria* females from all other species in the first bifurcation. The second bifurcation separated *H. nobile* from the honeybee workers, the beewolves, and *H. rutilans*. The remaining individuals were subdivided by bifurcation three into the honeybees on the one side and the beewolves and *H. rutilans* on the other side. Among beewolves and *H. rutilans*, however, the distinction was less clear-cut. The fourth bifurcation separated a group consisting of all male beewolves and four beewolf females (that shared (*Z*)-9-C25:1 as the major component) from a group consisting of the other three beewolf females (that shared (*Z*)-9-C27:1 as the major component) and *H. rutilans* females. Thus, the cuckoo wasps most closely resemble beewolf females exhibiting (*Z*)-9-C27:1 as their major cuticular compound. Only bifurcation five separated the *H. rutilans* females from the three beewolf females of that cluster. According to this analysis, *H. rutilans* females are considerably more similar to beewolf females than to females of the closely related *H. nobile*, and are about as similar to beewolf females as beewolf males.

The discriminant analysis, that followed the principal component analysis, calculated five discriminant functions that resulted in a complete separation of the six groups (Wilk's $\Lambda < 0.001$, d.f. = 40, $P < 0.001$; Fig. 8.3, Table 8.2). Discriminant function 1 represented 75.8 % of the variance and clearly separated females of both *C. arenaria* and *H. nobile* from the other groups. Discriminant function 2 represented 10.8 % of the variance and separated honeybees from a group consisting of male and female beewolves and *H. rutilans* females. Discriminant functions 3 (10.1% of the variance) and 4 (2.2 % of the variance) separated beewolf males from beewolf females. Only discriminant function 4 and discriminant function 5 (1.1 % of the variance) separated beewolf females from *H. rutilans* females. Thus, although *H. rutilans* females can be separated from beewolf females using GC-MS and discriminant analysis, the amount of variance that allows this separation is very small.

Table 8.1: Relative peak area of cuticular compounds: Relative peak area (in percent, not transformed) of compounds on the cuticle of *A. mellifera* workers (N = 8), *P. triangulum* males (N = 8), *P. triangulum* females (N = 7), *H. rutilans* females N = 13), *H. nobile* females (N = 4), and *C. arenaria* females (N = 4).

	A. mellifera workers	*P. triangulum* males	*P. triangulum* females	*H. rutilans* females	*H. nobile* females	*C. arenaria* females
C21	0.399	-	-	-	0.194	-
C22	0.128	-	-	-	-	-
(Z)-11-Eicosen-1-ol	0.471	-	-	-	-	-
(Z)-9-C23:1	2.81	0.261	-	-	0.884	-
(Z)-7-C23:1	0.301	-	-	-	0.210	-
C23	22.4	16.5	10.2	10.1	3.56	0.138
11-,9-MeC23	-	-	-	-	2.70	-
7-MeC23	-	-	-	-	2.69	-
5-MeC23	-	-	-	-	0.289	-
3-MeC23	-	-	0.387	1.10	1.01	-
C24:1	1.15	0.261	-	-	-	-
C24	0.508	0.756	-	-	0.213	0.188
(Z)-9-C25:1	5.31	34.5	40.8	11.0	9.55	0.038
(Z)-7-C25:1	0.233	-	-	10.6	3.10	0.087
C25	24.8	9.49	5.80	20.3	21.0	32.7
13-,11-,9-MeC25	0.043	-	-	1.95	9.41	1.22
7-MeC25	0.010	-	-	0.975	8.33	0.188
5-MeC25	-	-	-	0.489	1.40	0.576
7,11-diMeC25, 3-MeC25	-	-	-	-	0.710	0.254
C26:1	-	0.281	0.443	0.211	-	-
C26	0.384	-	-	-	0.764	0.531
13-,12-,11-,10-,9-,8-,7-MeC26	-	-	-	-	0.639	0.370
16-Pentacosen-8-one	-	-	0.307	-	-	-
(Z)-9-C27:1	1.93	2.16	31.2	13.9	0.683	0.434
(Z)-7-C27:1	-	-	-	6.32	0.588	5.42
C27	12.9	5.15	2.51	10.6	10.9	7.12
13-,11-,9-,7-MeC27	0.368	-	-	1.58	15.0	25.7
5-MeC27	-	-	-	-	0.311	0.073
9,13-diMeC27	-	-	-	-	0.412	0.320
7,11-diMeC27, 3-MeC27	-	-	-	-	-	0.223
5,9-,5,11-diMeC27	-	-	-	-	-	0.643
C28	0.154	-	-	-	0.249	0.197
14-,13-,12-,11-,10-,9-,8-,7-MeC28	-	-	-	-	0.286	0.546
18-Heptacosen-10-one	-	-	0.527	-	-	-
(Z)-9-C29:1	0.341	0.047	1.38	0.571	-	0.771
7-C29:1	0.653	-	-	-	-	5.00
C29	4.53	3.49	4.01	8.36	1.79	4.00
13-,11-,9-,7-MeC29	0.250	-	-	-	2.42	11.9
5-MeC29	-	-	-	-	-	0.247
7,11-diMeC29+3-MeC29	-	-	-	-	-	0.031
5,13-,5,11-,5,9-diMeC29	-	-	-	-	-	0.083
C30	-	-	-	-	-	0.055
14-,13-,12-,11-,10-,9-,8-MeC30	-	-	-	-	-	0.080
C31:1	4.78	0.553	-	-	-	0.046
C31:1	0.635	0.491	-	-	-	0.098
C31	5.41	1.85	2.47	1.98	--	0.246
15-,13-,11-,9-,7-MeC31	-	-	-	-	0.635	0.490
C33:1	0.762	-	-	-	-	-
C33:1	7.87	11.7	-	-	-	-
C33	0.377	12.6	-	-	-	-

Table 8.2: Group centroids for the discriminant analysis: Given are the respective group means of the five discriminant functions (percent explained variance).

	discriminant function				
species	1 (75.8 %)	2 (10.8 %)	3 (10.1 %)	4 (2.2 %)	5 (1.1 %)
H. rutilans females	-4.239	1.064	-3.980	2.312	-1.359
P. triangulum females	-6.591	2.100	-3.512	-0.187	3.498
P. triangulum males	-5.507	0.170	-1.122	-4.593	-1.205
A. mellifera workers	-6.414	-7.978	6.651	0.874	0.283
C. arenaria females	41.874	-3.566	-2.335	-0.332	0.316
H. nobile females	7.279	12.050	10.360	0.583	-0.175

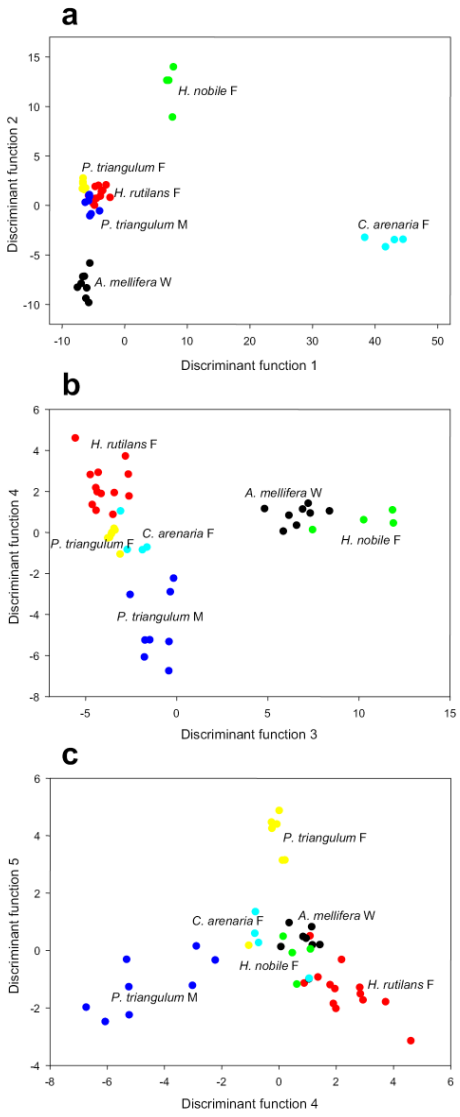

Fig. 8.3: Discriminant analysis of the cuticular compounds. Included are individual *P. triangulum* females (yellow), *P. triangulum* males (blue), *H. rutilans* females (red), *A. mellifera* workers (black), *C. arenaria* females (turquoise), and *H. nobile* females (green): Representation of the six groups for five discriminant functions a: Discriminant functions (DF) 1 and 2; b: DF 3 and 4; c: DF 4 and 5. The analysis is based on 8 factors revealed by the principal component analysis.

8.4 DISCUSSION

Beewolf females often attacked and evicted *H. rutilans* when they encountered them in front of their nest. This seems to be the rule for interactions between hosts and chrysidids although Linsenmaier (Linsenmaier 1997) reported that there are also cases where chrysidids do not elicit antagonistic behavior by their hosts. Prolonged stays at hosts' nests as observed in *H. rutilans*, have also been reported for other chrysidids (Kunz 1994; Linsenmaier 1997; Rosenheim 1987). Staying in vicinity of the host nest might allow the chrysidids to adjust the timing of oviposition to the most suitable stage of the provisioning cycle or to enter the nest in the absence of the host female. That *H. rutilans* females placed themselves under some cover (e. g. leaves) during prolonged stays might, besides the reduction of water loss, represent an attempt to hide themselves from the host females. Other chrysidid species also seem to hide near the entrance of a host nest and inspect the nest or brood cell after the host female has deposited provisions and departed for a new foraging flight (Linsenmaier 1997). This suggests that, similar to beewolves, most host species might recognize cuckoo wasps visually outside the nest. Since most chrysidids are brightly colored (see e.g. drawings in Linsenmaier 1997) this is not surprising.

However, there was no evidence that *H. rutilans* females were recognized by beewolf females in their nests although the nest owners approached the chrysidids several times. In contrast to Olberg's few reported cases (1953), we could never observe that chrysidids were thrown out of the nest by beewolf females neither in the study population in the field, nor in observation cages in the laboratory, nor during prolonged observations of beewolf nest aggregations as part of another study (Strohm et al. 2001). Possibly, different populations show varying local adaptations in the defense of the parasitoid or this behavior is extremely rare. However, we can not exclude that the chrysidids that Olberg (Olberg 1953) saw were not *H. rutilans*. Anyhow, our observations suggest that the *H. rutilans* females are often not detected in the nests. This finding is supported by the recognition bioassay. Filter paper discs treated with an extract of *H. rutilans* were recognized significantly less frequently than paper discs treated with an extract of the "alien" cuckoo wasp *C. viridula*. This suggests that the properties of the cuticular hydrocarbons of *H. rutilans* allow the cuckoo wasp to avoid recognition either when encountered inside the nest or after visiting the nest for inspection or oviposition. Our observations furthermore suggest that in the vast majority of cases *H. rutilans* females enter the nest when the female is out foraging and that they are only rarely directly encountered by the nest owner. Thus, it seems most important not to leave traces in the burrow during inspection of the nest or oviposition.

There are numerous reports of parasites of social species that gain access to their hosts' nests and protection from attacks mainly by chemical camouflage and more rarely chemical mimicry (Akino et al. 1999; Allan et al. 2002; D'Ettorre et al. 2002; Dettner & Liepert 1994; Lenoir et al. 2001; Martin et al. 2001; Moritz et al. 1991; see also Schiestl et al. 2000; Schiestl et al. 1999 for examples of chemical mimicry of sex pheromones in sexually deceptive orchids; Stowe 1998). Hydrocarbons are considered to represent the principal cues for nestmate recognition in social bees and wasps (Lenoir et al. 2001) and are most probably also involved in nest identification and species recognition in solitary species (Singer 1998). In our analysis, the GC-MS profiles of the cuticular hydrocarbons of *H. rutilans* and beewolf females show considerable similarity. At least, the null hypothesis based on the phylogenetic relationship of a closer resemblance between the two *Hedychrum* species as well as the two philanthine species was clearly contradicted. Our preliminary analysis of other species of chrysidids apart from *H. nobile* showed also distinct differences to the profile of *H. rutilans* (J. Kroiss, T. Schmitt, P. Schreier, E. Strohm, unpubl. data). Furthermore, other aculeate Hymenoptera show considerably different compositions of cuticular compounds (e.g. Butts et al. 1993; Butts et al. 1991; Hadley 1981; Howard & Blomquist 2005). This clearly contradicts a general similarity among all Hymenoptera or all aculeates or between chrysidids and crabronids. The profiles of the *H. rutilans* females were as close as or even closer to beewolf females than the profiles of beewolf males. Together with the behavioral tests, this provides strong evidence that *H. rutilans* females are chemically cloaked. This will help to reduce the probability of detection during or –more importantly- after their presence in the nest.
A priori, chemical camouflage and mimicry seem unlikely to evolve in a chrysidid wasp that attacks a solitary host. Chemical camouflage, i.e. the acquisition of mimetic compounds from a solitary host by a parasitic species, might be problematic since, in contrast to parasites of social hosts, there is little opportunity to sequester cloaking chemicals. Social host species possess large nests and a large number of colony members that might serve as sources for the relevant compounds. Brood parasites of solitary brood caring Hymenoptera have rarely been studied in detail (e.g. Hefetz et al. 1982). The only example of chemical camouflage in a brood parasite of a solitary species comes from *Nomada* bees. In some species of this genus, females have been reported to acquire mimetic odors by being perfumed by males during mating. Females of these species seemed not to elicit aggressive responses when encountered by host females of the genus *Andrena* (Tengo & Bergstrom 1977). Chemical cloaking in chrysidid wasps has not yet been reported.

With chemical camouflage being an unlikely option for a brood parasite of solitary species, chemical cloaking might evolve by synthesis of the compounds, i.e., chemical mimicry. For most chrysidids this is also unlikely since they attack a large number of different host species (e.g. Kunz 1994) with a varying composition of cuticular chemicals that is not compatible

with an efficient chemical cloaking. However, *H. rutilans* is *de facto* monospecific in the study area and is, thus, predestined to evolve chemical mimicry. The behavioral observations show that cuckoo wasps do not regularly stay in nests for long periods. This makes a sequestration of host chemicals that are only available in relatively small amounts at the walls of the burrow rather unlikely. The assumption that the cloaking compounds are synthesized by *H. rutilans* females themselves is supported by details of the composition of chemicals on their cuticle. The occurrence of considerably proportion of the (*Z*)-7 isomers of the respective major components of the beewolf cuticle ((*Z*)-9-C25:1 or (*Z*)-9-C27:1) in the brood parasitoid but the lack thereof (at least in comparable proportions) in beewolf females contradicts an acquisition of the chemicals from their host. Thus, most probably cuckoo wasps produce at least some of the compounds on their cuticle by themselves.

In addition to the qualitative imitation of the hydrocarbons of its host, the cuckoo wasps might also employ a quantitative strategy to evade detection by beewolf females. Preliminary data suggest that *H. rutilans* (as well as several other chrysidids studied by us) have an overall low level of cuticular substances (Kroiss, Schmitt, Strohm, unpubl. data). Such a reduction in the amount of cuticular hydrocarbons as a means to avoid detection by the host has been suggested by Jeral et al. (1997) in thievery ants (see also Lenoir et al. 2001). In fact, the background against which the cuckoo wasp is perceived (or rather not perceived) by the beewolf female is the wall of the nest burrow. This is contaminated with compounds from the cuticle of the nest owner due to the contact with its mandibles during excavation and with the tarsi and abdomen during processing of the excavated sand and movement inside the nest. Preliminary analyses suggest that the typical beewolf cuticular hydrocarbons can be sampled from the nest walls (Kroiss and Strohm, unpubl. data). Thus, the chemical traces or the presence of a cuckoo wasp whose hydrocarbon profile is sufficiently similar to the background might not be recognized by a beewolf female.

8.5 CONCLUSIONS

H. rutilans might employ a combination of strategies to evade detection. If encountered in the nest, they run away or remain motionless. They possibly leave only very small amounts of decisive and traitorous substances in the nest. Most notably, the composition of their cuticular hydrocarbons is very similar to that of their host. Thus, *H. rutilans* females seem to be able to avoid detection when directly encountered by a beewolf female in the nest. Much more important, however, is the reduction of the conspicuousness of scent marks left in the

nest burrow or on the bee during oviposition. This is to our knowledge the first reported evidence for chemical mimicry (sensu Dettner & Liepert 1994) in a parasitoid of a solitary wasp.

8.6 MATERIALS AND METHODS

8.6.1 Behavioral observations

8.6.1.1 Behavior outside the nest

We observed interactions between cuckoo wasps (*H. rutilans*) and beewolf females in the field in a beewolf nest aggregation on the Campus of the University of Würzburg. Over several years there were about 100 – 500 beewolf nests (easily detectable due to the characteristic nest mounds) and 50 – 500 *H. rutilans* females (determined by capture-mark-recapture methods (Mühlenberg 1993, E. Strohm, unpubl. data)). Behavioral interactions between beewolf females and cuckoo wasps at 24 focal nests (located on an area of about 10 x 5 m) were recorded for a total of 54 hours. We observed whether beewolf females showed any signs of disturbance or agonistic behavior when a cuckoo wasp was present in the vicinity of their nests. We recorded the following behaviors of cuckoo wasps and beewolf females: 1. Landing of *H. rutilans* on nest mound. 2. Time it stayed on nest mound (for stays > 4 sec.). 3. Whether the nest was open or closed. 4. Whether the cuckoo wasp entered the nest. 5. The time the cuckoo wasp stayed in the nest and whether the nest owner was at home or not. 6. Behavior of the cuckoo wasp during its stay outside the nest (running, sitting hiding, no exact durations were recorded). 7. Whether the cuckoo wasp tried to oviposit on a bee when a female returned with prey. 8. Whether and how a female responded to the presence of a cuckoo wasp when returning with prey.

8.6.1.2 Behavior inside the nest

8.6.1.2.1 Experiment I

The interaction between host and parasite inside the nest was investigated using observation cages in the laboratory (for details see Strohm 1995). These cages allow observation of the behavior of host and parasite in the main burrow. Beewolf females (N = 6), either from the

laboratory population or from the field were kept individually in such cages and one cuckoo wasp that was caught in the field was introduced per cage (overall 7 *H. rutilans* females were used). Honey was provided *ad libitum* for both species. Honeybees were also provided *ad libitum* as prey for the beewolves. Since a pilot study revealed that the cuckoo wasps need a humid retreat, petri-dishes with a layer of moist sand and gravel were placed into the flight compartment of each cage and moistened daily. Observations of interactions in the nest burrow were carried out under dimmed red light that did not elicit any disturbance in either species. If a cuckoo wasp and a female were in a nest at the same time, we recorded the behavior of both.

8.6.1.2.2 Experiment II

To assess the significance of the cuticular hydrocarbons for the detection of *H. rutilans* beewolf female inside the nest, we established a recognition bioassay. We recorded the females' response towards paper discs treated with extracts. To make sure that beewolf females responded to the paper discs at all, we needed a positive control, i.e., extracts from a species that was recognized as an intruder and elicited strong responses by beewolf females. We used extracts of another chrysidid, *Chrysis viridula* (although it might have been preferable to use extracts of *H. nobile*, fresh specimens of this species were not available due to their rarity). *C. viridula* is a parasitoid of eumenid wasps and it may occur in the same habitat as *H. rutilans*. Its cuticular hydrocarbons differ considerably from the composition of the cuticular hydrocarbons of *H. rutilans* (Kroiss and Strohm, unpubl. data). Both cuckoo wasp species are very similar in size and the total amount of their cuticular hydrocarbons is alike (J Kroiss, E. Strohm, unpublished data). Cuticular hydrocarbons of females of *H. rutilans* and *Chrysis viridula* were extracted for 10 min in 0.5 ml distilled n-hexane (Fluka). The hexane of the cuticular extracts was evaporated under a stream of nitrogen at ambient temperature and the extract was redissolved in 100µl hexane. An aliquot of 10µl of an extract was applied onto a circular filter paper disc (diameter 6mm. *H. rutilans*: N = 7, *C. viridula*: N = 8) and the solvent was allowed to evaporate for 5 min. As a negative control 10µl of pure hexane were applied onto a filter paper disc and evaporated (N = 7). The filter paper discs were inserted into the main burrow of individual beewolf nests (N = 9) in observation cages (see above) one after the other in a randomized sequence. After introduction of the filter disc we continuously recorded the behavior of individual beewolf females using a voice recorder until the paper disc was evicted from the nest or until it was incorporated into excavated material in the nest. Beewolf females biting a paper disc or alert freezing directly at the paper disc when approaching it at any time during a trial were considered as evidence that a female has recognized the paper disc as something that differed from the background odor. The

freezing behavior was distinctive and could not be observed as a spontaneous behavior. If one or several of these behaviors occurred the respective trials was considered as showing a response by the beewolf female. In contrast, trials during which females were walking over the paper disc without any response were classified as "no recognition". The prediction that extracts of *H. rutilans* elicited weaker responses than the positive control and similar responses as the negative control was tested by comparing the number of trials with and without recognition. The small sample size necessitated using Fisher's exact tests.

8.6.2 Chemical analyses

We caught females of *H. rutilans* (N = 13) in the vicinity of beewolf nests on the campus of the University of Würzburg. Beewolf females (N = 7) and males (N = 8) were taken from the same field site in Würzburg or from a laboratory population that was bred from the same population. Honeybee foragers (*Apis mellifera carnica*) (N = 8) were caught from hives in the vicinity of the field site when leaving the nest. Females of *Hedychrum nobile* (Hymenoptera, Chrysididae) (N = 4) and its host, *Cerceris arenaria* (Hymenoptera, Crabronidae, subfamily Philantinae) (N = 4) were caught at a nesting aggregation near Vizzola Ticino, Italy. These two species are another host-parasitoid pair and served as an outgroup for *H. rutilans* and *P. triangulum* to control for phylogenetic relationships. All individuals were killed by freezing (1 h, –20 °C). Chemicals on their cuticles were extracted for 10 min in 0.5 ml distilled n-Hexane (Fluka).

8.6.2.1 Capillary Gas chromatography – Mass spectrometry

Capillary gas chromatography-mass spectrometry (GC-MS)-analysis was performed with a Fisons Instruments (Fisons, Engelsbach, Germany) GC 8000 Series coupled to a Fisons Instruments MD800 quadrupol mass detector. We used a DB-5MS fused silica capillary column (30 m x 0.25 mm i.d.; df = 0.25µm) (J & W, Folsom, CA, USA). The GC was programmed from 60° C for 1 min then to 310° C for 10 min with a temperature increase of 5°/min, with 2 ml/min flow rate of helium gas. We chose a splitless injection mode (1µl) at an injector temperature of 250° C and a splitless period of 60 sec. The mass spectrometer was operated in EI mode at 70 eV. The software Xcalibur for Windows was used for data acquisition.

8.6.2.2 Capillary Gas Chromatography – Fourier Transform Infrared Analysis (HRGC-FTIR)

HRGC-FTIR spectra were obtained using an HP 5890 GC (Agilent Technologies, Böblingen, Germany) coupled to an FTS 575C Tracersystem (BioRad, Hercules, CA, USA). GC separation was performed using a DB-1 capillary column (30 m x 0.25 mm ID; df = 0.25 µm; J & W Scientific, Folsom, CA, USA). Temperature was programmed from 80 to 270°C with 4°C/min heating rate. Helium was used as carrier gas with a constant flow of 1–2 ml/min. Injection was carried out using a split/splitless injector at 250°C in the splitless mode for 60 sec. Injection volume was 0.1 µl. IR spectra were recorded by scanning 256 times in a frequency range from 4000 to 700 cm^{-1} with a resolution of 1 cm^{-1}. Data system was a Dell Optiplex GX110-PC with BioRad WinIR Pro (Version 2.7) Tracer Software and Sadtler IRSearchMaster.

8.6.2.3 Compound identification

The chemical structure of the components of the cuticular hydrocarbons was determined by comparing retention times and diagnostic ions of the mass spectra with purchased chemicals and the use of a commercial MS database (NIST 4.0). Methylalkanes were characterized using diagnostic ions and by determining Kovats indices according to the method of Carlson et al. (1998). The position of double bonds was determined by DMDS derivatisation (Dunkelblum et al. 1985). The configuration of double bonds was revealed by HRGC-FTIR (Attygalle et al. 1994; Schmitt et al. 2003). Some components could not be identified and for some alkenes the position of the double bond and its configuration could not be determined due to the small amounts on the cuticles. However, neither of the unidentified components occurs on beewolf females and cuckoo wasps. Thus, they do not confound the similarity between these two groups that are most important to our question. The alkenes listed in one line in Table 8.1 as the same compound for beewolves, cuckoo wasps and honeybees are most probably identical since their mass spectra and the retention times are identical. Thus, the comparison between beewolf females and cuckoo wasps is not confounded by the incompletely identified alkenes.

8.6.3 Data analysis

The results of the behavioral observations are given as the mean ± SD and/or the median. For the recognition bioassay, we compared the number of individuals showing a response

facing the paper disc between the treatments. Different treatments were compared using Fisher's exact test (two-tailed) using the program BIAS for Windows version 8.2 (epsilon-Verlag GbR, H. Ackermann, Frankfurt/Main, Germany). Since some of the females died before their response to all stimuli could be tested, sample sizes differ between the three stimuli.

Patterns of chemicals on the cuticle were analyzed by multivariate methods. Since we were interested in the similarity between beewolf females, beewolf males, honeybee workers, *H. rutilans* females, and *H. nobile* and *C. arenaria* females, we performed a hierarchical cluster analysis to assess the pattern of similarity without *a priori* grouping. Furthermore, we conducted a discriminant analysis to test whether the groups are separated by discriminant functions. Due to the large number of peaks relative to the sample size the discriminant analysis might lead to confounded results with regard to the hypothesis tested. Thus, we reduced the number of variables for the discriminant analysis using principal component analysis (varimax rotation, eigenvalues > 1; 8 variables were extracted that represented 89 % of the variance of the total sample).

Since relative peak areas of a sample are not statistically independent we transformed the data according to Aitchison (1986) see e.g. (Nielsen et al. 1999). However, the original transformation procedure makes it necessary to exclude compounds that do not occur in all samples. Thus, peaks that are zero in some samples but are present in other samples would not have been considered. When analyzing whether groups can be separated by their profiles such a procedure is conservative. However, for the aim of this study, the exclusion of peaks that are not present in all samples would have erroneously increased the similarity between the groups and, thus, confounded the result. Therefore, we modified the transformation to avoid undefined values for peaks with an area of zero (log10((relative peak area/geometric mean relative peak area)+1)). The resulting variables were normally distributed. We used the squared Euclidean distance as a measure of distance for cluster analysis and between groups average linkage as the method for combining clusters. Analyses were calculated using SPSS 13.0.1.

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CHAPTER 9

CHEMISCHE TARNKAPPE BEI EINEM BRUTPARASITEN DES BIENENWOLFS.

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9.1 ZUSAMMENFASSUNG

Wenn das Weibchen eines Bienenwolfs eine gelähmte Biene in ihre Bruthöhle einträgt, wird sie oft von brutparasitischen Goldwespen heimgesucht. Deren Weibchen haben es auf die erbeutete Biene abgesehen, um darauf ihr Ei abzulegen. Hat eine Goldwespe damit Erfolg, so schlüpft deren Larve und frisst nicht nur die eingetragenen Bienen, sondern auch die Larve des Bienenwolfs. Nun wurde gezeigt, dass diese auf Bienenwölfe spezialisierte Goldwespe den Geruch ihres Wirtes imitiert, also eine „chemische Mimikry" anwendet, um möglichen Abwehrmaßnahmen des Bienenwolfs zu entgehen.

9.2 EINLEITUNG

Interaktionen zwischen Wirten und ihren Parasiten oder Parasitoiden stellen wichtige Triebkräfte der Evolution dar (Godfray 1994). Wirtsarten sollten Mechanismen entwickeln, die es ihnen erlauben, ihre Feinde zu entdecken und entsprechende Abwehrmaßnahmen (z.B. Töten des Parasiten oder Entfernen der parasitischen Eier) zu ergreifen. Parasiten oder Parasitoide hingegen sollten Wege finden, einer Entdeckung durch ihre Wirte zu entgehen. Ist der Parasit auf eine einzige Wirtsart spezialisiert, kann es durch ständige Anpassungen und Gegenanpassung zu einem evolutionären Wettrüsten mit den Wirten kommen.
Insbesondere Hautflügler, die Brutpflege betreiben, sind häufig das Ziel parasitischer Insekten. Diese haben es auf die Brut selbst oder deren Nahrungsvorrat, meist Pollen oder

erbeutete Insekten, abgesehen. Um diese Nahrungsquelle zu erreichen, müssen sie in das Wirtsnest eindringen. Dabei sollten sie möglichst wenig Spuren hinterlassen, um von den Wirten nicht entdeckt und angegriffen zu werden.

Verräterisch können vor allem chemische Stoffe sein, denn diese spielen in der Kommunikation von Insekten eine große Rolle (Godfray 1994; Jackson & Morgan 1993). Für die Arterkennung sind die kutikulären Kohlenwasserstoffe von entscheidender Bedeutung. Dies sind Substanzen auf der Oberfläche, die primär dem Verdunstungsschutz dienen. Ihre Zusammensetzung variiert stark zwischen verschiedenen Arten. Parasiten, deren kutikulären Kohlenwasserstoffe denen ihrer Wirte ähneln, sollten somit eine „chemische Tarnkappe" tragen, die ihnen zumindest in der Dunkelheit eines Nestes Schutz gewährt. Eine solche chemische Tarnung hat man bei mehreren Parasiten von Ameisen tatsächlich nachgewiesen. Diese schützen sich, indem sie die fraglichen Substanzen direkt vom Wirt oder ihrem Nestmaterial aufnehmen. Eine chemische Mimikry mit selbst synthetisierten Kohlenwasserstoffen, also eine echte Nachahmung, ist hingegen sehr viel aufwendiger, denn hierzu muss der Stoffwechsel des Parasiten eingestellt sein. Es ist daher nicht verwunderlich, dass diese Art der Tarnung bisher nur selten nachgewiesen wurde (Dettner & Liepert 1994).

Ein solch ungewöhnlicher Fall wurde nun für die Goldwespe *Hedychrum rutilans* beschrieben. Diese hochgradig wirtsspezifische Art lauert den Weibchen des Europäischen Bienenwolfs auf. Die Weibchen dieser Grabwespe jagen Honigbienen und tragen sie als Futter für ihre Larven in das Nest ein. Diese als Futtervorrat vorgesehenen Bienen sind das Ziel der weiblichen Goldwespe. Entweder lauert sie einem Bienenwolfweibchen am Nesteingang auf und versucht dort, ihr Ei rasch auf die Beute abzulegen, oder sie dringt später in das Nest ein und versucht eine dort deponierte Biene mit einem Ei zu beschicken. Letzteres ist die bei weitem häufigere Methode. Gelegenheit für eine Eiablage im Nest ist gegeben, denn es werden zunächst mehrere Bienen in das Nest eingetragen und zwischengelagert, ehe das Bienenwolfweibchen ein Ei auf eine der Bienen legt und dann die Brutzelle verschließt.

Die Goldwespenlarve schlüpft früher als die des Bienenwolfs, tötet diese und ernährt sich dann von den eingetragenen Bienen. Der Schaden, der den Bienenwölfen durch die brutparasitischen Goldwespen entsteht, kann enorm hoch sein: zwischen 3 und 30% der Brutzellen einer Bienenwolfpopulation können befallen sein (Strohm et al. 2001). Da die Bienenwolfweibchen Goldwespen und mit einem fremden Ei versehene Bienen aus dem Nest werfen können, hängt der Erfolg der Goldwespen in hohem Maße davon ab, dass sie und die von ihnen abgelegten Eier nicht von ihren Wirten entdeckt werden.

9.3. ERGEBNISSE UND DISKUSSION

Chemisch-analytische Untersuchungen mittels Gaschromatographie und Massenspektrometrie zeigten nun, dass die Goldwespenweibchen den Geruch ihrer Wirte nachahmen (Strohm et al. 2008). Zwar gibt es geringfügige Unterschiede in der Zusammensetzung der kutikulären Kohlenwasserstoffe beider Arten, jedoch sind die Goldwespen den Bienenwolfweibchen sogar ähnlicher als Bienenwolfmännchen und weitaus ähnlicher als Honigbienen. Stammesgeschichtlich sind Bienenwölfe mit Bienen viel enger verwandt als mit Goldwespen. Man sollte also erwarten, dass Bienen und Bienenwölfe in dieser Hinsicht am ähnlichsten sind. Dass dem nicht so ist, spricht dafür, dass erhebliche Selektionsdrücke auf die chemische Angleichung des Parasitoids an den Wirt wirksam sind.

Ob Bienenwolfweibchen tatsächlich auf die chemische Täuschung der Goldwespen hereinfallen, wurde in Verhaltenstests untersucht. Dazu wurden Filterpapierscheiben mit dem Geruch einer anderen, nicht bei Bienenwölfen parasitierenden, Goldwespenart (*Chrysis viridula*) behandelt und ins Nest eines Bienenwolfweibchens gelegt. Diese Filterpapiere wurden von den Bienenwolfweibchen beinahe immer erkannt und oft heftig "angegriffen". Filterpapiere mit Geruchsextrakten von *H. rutilans* jedoch wurden, genauso wie unbehandelte Filterpapiere, praktisch nie entdeckt und entgingen so den "Angriffen" durch die Wirte (Strohm et al. 2008). Diese Ergebnisse zeigen, dass die chemische Ähnlichkeit der Goldwespen mit den Bienenwolfweibchen das Risiko einer Entdeckung und Abwehr der Goldwespen durch ihre Wirte stark verringert.

Während eine solche chemische Mimikry von einigen Parasiten sozialer Insekten wie Ameisen oder Wespen bereits bekannt ist (Dettner & Liepert 1994), ist dies der erste Nachweis einer chemischen Tarnung bei einem Parasitoiden eines solitären Hautflüglers. Wie bereits erwähnt bedienen sich die Parasiten sozialer Insekten der bereits vorliegenden fremden chemischen Stoffe, die in größeren Nestern mit zahlreichen Kontaktmöglichkeiten mit Nestbewohnern und Nestmaterial leicht aufzunehmen sind. Im Nestmaterial der Bienenwölfe sind die typischen kutikulären Kohlenwasserstoffe zwar auch vorhanden, aber die Menge reicht für eine chemische Camouflage der Goldwespen vermutlich nicht aus. Außerdem weisen Details der Zusammensetzung der kutikulären Kohlenwasserstoffe der Goldwespen darauf hin, dass diese von den Goldwespen selbst synthetisiert und nicht vom Nestmaterial aufgenommen werden. Somit gibt es deutliche Hinweise, dass die Goldwespe eine echte chemische Mimikry entwickelt hat.

Durch die Nachahmung der kutikulären Kohlenwasserstoffe ihrer Wirte, der Weibchen des Europäischen Bienenwolfs, besitzt die Goldwespe *H. rutilans* gewissermaßen eine chemische Tarnkappe, die es ihr erlaubt, einer Erkennung und Abwehr durch die Bienenwölfe zu entgehen.

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CHAPTER 10

LOW LEVEL OF CUTICULAR HYDROCARBONS IN A PARASITOID OF A SOLITARY DIGGER WASP AND ITS POTENTIAL FOR CONCEALMENT

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10.1 SUMMARY

Insect cuticular hydrocarbons (CHCs) play a role as semiochemicals in many host parasite systems and chemical mimicry or camouflage is a well-known mechanism of parasites to evade detection by the host. The cuckoo wasp *Hedychrum rutilans* (Hymenoptera, Chrysididae) is a parasitoid of larvae of the European beewolf *Philanthus triangulum* (Hymenoptera, Crabronidae). Females chemically mimic the cuticular hydrocarbons of their hosts to avoid detection and countermeasures when entering the host nest for oviposition. Here we report on a possible second mechanism of the chrysidid wasp *H. rutilans* to evade detection: the amount of CHCs per mm² of cuticle is only about 1/5 compared to its beewolf host. Furthermore, we show that surprisingly large amounts of CHCs of beewolf females can be found on the walls of the underground nest. Potentially, these hydrocarbons might constitute a background odor against which the cuckoo wasps or their chemical traces have to be perceived by the beewolf. The reduction in the amount of CHCs of the cuckoo wasps might be equivalent to a dilution of recognition cues, especially against the background odor of the nest walls, and might provide a means to escape detection within the nest due to "chemical insignificance".

10.2 INTRODUCTION

The cuticle of insects is covered with hydrocarbons (CHCs) that serve to decrease water loss (Gibbs 1998; Lockey 1988). Besides this primary function, insect CHCs have been shown to represent semiochemicals for species recognition (Howard 1982; Howard & Blomquist 2005; Lucas *et al.* 2005; Singer 1998) as well as kin and nestmate recognition (Dapporto *et al.* 2006; Ruther *et al.* 2002; Singer 1998). Due to this function of CHCs as infochemicals they play a crucial role for the detection of any (social) parasites or parasitoids that invade the nest to parasitize the brood or steal resources (Howard & Blomquist 2005). While host species should evolve effective countermeasures upon recognition of parasites or their eggs, parasites in turn are selected to evade detection by the host. This can be achieved by chemical cloaking. This is accomplished by either imitation (chemical mimicry) or acquisition (chemical camouflage) of the host CHC profile by the parasite (Dettner & Liepert 1994). Most examples of chemical deception consist of camouflage and are found in parasites of social Hymenoptera (Allan *et al.* 2002; D'Ettorre *et al.* 2002; Dettner & Liepert 1994; Martin *et al.* 2001; Moritz *et al.* 1991; Vander-Meer & Wojcik 1982). Apart from parasites of social insects, chemical mimicry has been shown e.g. for predators and parasitoids of ant-attended aphids (e.g. Liepert & Dettner 1996; Lohman et al. 2006) and for a cleptoparasitic tenebrionid beetle (Geiselhardt et al. 2006).

Recently, the first example of chemical mimicry of a parasitoid of a solitary wasp has been described (Strohm et al. 2008). The cuckoo wasp *Hedychrum rutilans* (Hymenoptera, Chrysididae) is a highly specialized brood-parasitoid of the European beewolf *Philanthus triangulum* (Hymenoptera, Crabronidae) (Kimsey & Bohart 1990; Strohm et al. 2008). Such a high degree of specialization in a host-parasite system facilitates the onset of an (co)evolutionary arms race with numerous reciprocal adaptations and counteradaptations of hosts and parasites, be it anatomical, physiological, or behavioral (Davies *et al.* 1989; Dawkins & Krebs 1979). Females of *H. rutilans* have to deposit their eggs on the host prey, namely paralyzed workers of the honeybee *Apis mellifera* that are temporarily couched in the main burrow of the host nest (Strohm *et al.* 2008; for a detailed description of the oviposition strategy of *H. rutilans* see Strohm *et al.* 2001). GC-MS profiles of CHCs of both species show considerable similarity and behavioral observations suggest that the composition of the CHCs of *H. rutilans* significantly reduces the probability that the cuckoo wasp or traces of its presence in the nest are detected by the beewolf female (Strohm et al. 2008). Thus, there is convincing evidence that chemical mimicry is employed by female *H. rutilans* to evade detection by its host.

So far, most of the studies on chemical mimicry focused on the qualitative composition i.e. the identity and relative proportions of the hydrocarbons of both host and parasite species (Akino *et al.* 1999; Allan *et al.* 2002; Bagneres *et al.* 1996). By contrast, there are few reports on the quantity of the parasites' CHCs in chemical mimicry systems (D'Ettorre & Errard 1998; Jeral *et al.* 1997; Lorenzi & Bagneres 2002). There is evidence that a low amount of CHCs, as observed in these studies, causes a dilution of recognition cues and represents a second strategy of these social parasites to evade detection by the host in the sense of a "cuticular chemical insignificance" (Jeral *et al.* 1997; Lenoir *et al.* 2001; Lorenzi *et al.* 2004; Panek *et al.* 2001). A reduction of CHCs of a parasitoid of a solitary host should be even more important compared to parasites of a social species, since the total level of CHCs ("background odor") in the nest of a solitary species is probably lower compared to social species due to the larger number of individuals active in a nest.

Here we test the hypothesis that *H. rutilans* females employ a second strategy by reducing the amount of CHCs to conceal the parasite or its traces in the nest and avoid defense by the host species. In order to assess the potential of a reduction in the amount of CHCs to reduce conspicuousness, we examine the background level of CHCs on the walls of the beewolf nest.

10.3 MATERIALS AND METHODS

10.3.1 Insects and sampling

Females of *H. rutilans* (N=9) were caught in the vicinity of beewolf nests at beewolf aggregations on the campus of the University of Würzburg and in a sandpit near Retzbach, Germany. Beewolf females (N=11) were taken from the same field site in Würzburg or from a laboratory population that was bred from the same population. After anaesthetizing the animals with CO_2, they were killed by freezing and kept frozen at –20°C until extraction of the CHCs and GC-MS analysis.

10.3.2 Sampling of nest wall material

Individual beewolf females were kept in observation cages as described before (Strohm & Linsenmair 1995). Nest wall material (i.e. sand) of European beewolves was obtained from 12 nests. The glass cover of the observation nests was lifted and a scoop was used to scrape sand

off the walls of the main burrow of the nest. 3 g of nest wall material (equivalent to 7 cm of nest burrow wall with about 1200 mm² of nest wall surface) were collected from each nest.

10.3.3 Chemical analysis

Frozen animals were thawed for 10 min prior to extraction. Thawed wasps were placed individually in glass vials (4 ml), and 5 µl (*H. rutilans*) or 10 µl (*P. triangulum*) of a 1 g/l solution of octadecane in hexane (equivalent to an amount of 5 or 10 µg of octadecane) was added as an internal standard to each vial to allow quantification of the CHCs. Then 0.5 to 1.0 ml of distilled n-Hexane (Fluka) was added and CHCs were extracted for 10 min. The hexane extracts were reduced in volume by a gentle stream of nitrogen at ambient temperature to a final volume of 50-100 µl.

Nest-wall samples were extracted for 10 min in 2.5 ml hexane in a 4 ml glass vial, and 5 µl of a 1 g/l solution of octadecane in hexane (equivalent to an amount of 5 µg of octadecane) was added as an internal standard. The hexane extracts were reduced in volume as described above to a final volume of 50-100 µl.

Samples were analyzed by coupled capillary gas chromatography-mass spectrometry (GC-MS) as described before (Strohm et al. 2008). The CHC peaks in the extracts of *P. triangulum*, *H. rutilans*, and the nest walls were manually integrated using the MSD ChemStation software (Agilent Technologies). The chemical structures of CHC components were determined as in earlier studies (Strohm *et al.* in press; Strohm *et al.* 2008) using retention times, diagnostic ions of the mass spectra, and a commercial MS database (NIST 4.0). Using the octadecane peak as an internal standard, the total amount of CHCs was calculated.

10.3.4 Calucalation of cuticular surface

To take the difference in body size of the two species into account, the cuticular surface of each individual was estimated using mathematical formulae for standard geometric shapes. For a calculation of the cuticular surface of the two species length, width, and height of head, thorax, and abdomen were measured to the nearest 1/10 mm using an electronic caliper under a dissecting scope. Since thorax and abdomen approximately resemble prolate ellipsoids, the surface of these body parts was calculated according to the formula

$$A = \frac{2\pi a^2 b}{\sqrt{a^2 - b^2}} \left[\frac{b}{a^2} \sqrt{a^2 - b^2} + \arcsin\left(\frac{\sqrt{a^2 - b^2}}{a} \right) \right]$$

with a being the semimajor axis (length/2) and b being the semiminor axis, calculated as (width + height)/4. The surface of the head was estimated according to its cylinder-like shape using the formula $A = 2\pi r(r + h)$ with h being the length of the head and r being (width + height)/4. The total surface of the animals was calculated by adding the surfaces of the three body parts. The overall shape of the body parts of both species is rather similar and both species have only few hairs on their body.

10.3.5 Data analysis

The total amount of CHCs was calculated and then $\log_{10}$-transformed to obtain normally distributed data for statistical analysis. The absolute amounts of CHCs as well as the total cuticular surface were compared between the two species by t-tests. The amount of CHCs per mm² was calculated for each individual by dividing the total amount of hydrocarbons by the respective cuticular surface. The CHC amount per mm² was also $\log_{10}$-transformed and compared between the two species by a t-test.

For the nest wall extracts, the total amount of CHCs per g nest material was calculated. For the estimation of the amount of CHCs per mm² of nest wall surface, we approximated the nest wall surface using the formula $A = \frac{2}{3} 2\pi r l$ with r being the radius of the nest burrow (3 mm) and l being the length of the sampled nest burrow (70 mm). About 1/3 of the burrow wall was formed by the cover glass plate of the observation cage. Therefore, we used only 2/3 of the total circumference of the burrow for the calculation of the nest wall surface. All values are given as the mean ± SD. SPSS 13.01 software was used for all calculations.

10.4 RESULTS

The CHC composition of *P. triangulum* and *H. rutilans* females was characterized by the presence of alkanes, alkenes, methylalkanes, and – in the case of the beewolves – of two ketones (see Strohm et al., 2008 for details). According to the difference in body size, the

total cuticular surface was significantly higher in *P. triangulum* (155.7 ± 22.1 mm²) compared to *H. rutilans* (46.2 ± 13.3 mm²; t-test, T = -13.009, df = 18, p < 0.001). The total amount of CHCs was 16.2 times higher in *P. triangulum* (188.4 ± 117.1 µg; minimum: 45.4µg, maximum: 376.0 µg) compared to *H. rutilans* (11.6 ± 9.9 µg; minimum: 1.80 µg, maximum: 35.5µg). This difference was highly significant (t-test, T = -8.014, df = 18, p < 0.001). Most important for our question, the amount of CHCs per mm² of cuticular surface in the beewolf females was by factor 4.6 higher than in the cuckoo wasp (*P. triangulum*: 1.15 ± 0.73 µg/mm²; *H. rutilans*: 0.25 ± 0.17 µg/mm²) and differed significantly (t-test, T = -4.065, df = 18, p < 0.01, Fig. 10.1).

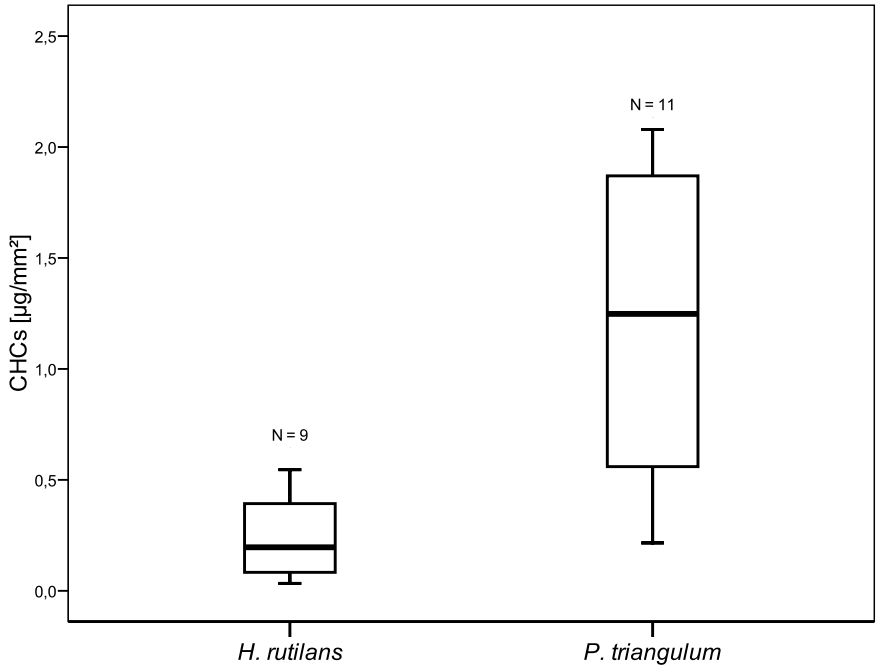

Fig. 10.1: Amount of cuticular hydrocarbons per mm² of body surface of *H. rutilans* and *P. triangulum* females. Bold lines represent medians, boxes comprise the interquartile range, and whiskers indicate minimum and maximum values. Quantities are given in µg of CHCs per mm² of body surface. The amount of CHCs per mm² of body surface differs significantly between species (data $\log_{10}$-transformed, t-test, T = -4.065, df = 18, p < 0.01).

In the nest-wall samples, we found the typical CHCs of beewolf females, though sometimes in low quantities. The main components of the hydrocarbons were (*Z*)-9- and (*Z*)-7-pentacosene, (*Z*)-9-heptacosene, and tricosane (Table 10.1). The total amount was 1.76 ± 0.76 µg CHCs per gram of nest wall material. The amount of CHCs per mm² of the nest wall was estimated as 6.14 ± 2.71 ng/mm².

Table 10.1: Chemical composition of the CHCs extracted from the wall material of *P. triangulum* nests (N = 12). Given are the means and standard deviations of the percentage of different compounds.

Compound	Mean	SD
Heneicosane	0.414	0.209
Docosane	0.645	0.380
(Z)-7-Tricosene	0.789	0.253
(Z)-9-Tricosene	0.271	0.136
Tricosane	12.4	4.22
11-, 9-, 7-Methyltricosane	0.063	0,090
5-Methyltricosane	0.039	0.092
Δ-x-Tetracosene	0.498	0.464
Tetracosane	2.00	1.38
(Z)-9- and *(Z)*-7-Pentacosene	23.8	17.3
Pentacosane	13.8	4.02
13-, 11-Methylpentacosane	0.093	0.107
(Z)-9-Hexacosene	0.759	0.698
Hexacosane	2.42	2.18
Δ-16-Pentacosen-8-one	1.77	1.03
(Z)-9-Heptacosene	16.0	16.9
Heptacosane	8.20	4.09
Octacosane	1.80	1.56
Δ-x-Heptacosenone	0.461	0.616
Δ-x-Nonacosene	1.17	0.783
Nonacosane	6.75	1.94
Triacontane	1.10	0.891
Δ-x-Hentriacontene	0.500	0.350
Δ-y-Hentriacontene	0.310	0.271
Triacontane	2.46	0.526
Dotriacontane	0.329	0.370
Δ-x-Tritriacontene	0.843	0.706
Tritriacontane	0.286	0.186

10.5 DISCUSSION

These results show that the cuckoo wasp *H. rutilans* bears considerably lower amounts of CHCs on its cuticle than its host species. Apart from imitating the CHC-composition of its host *P. triangulum*, as has been shown earlier (Strohm et al. 2008), this reduction of the CHCs might be equivalent to a dilution of recognition cues. Although the amount of CHCs per mm² of the nest wall, representing a background odor, is considerably lower compared to the cuticle of the cuckoo wasps, this reduction of recognition cues might impede the detection of

the parasitoid or its traces by its host. At least detection of the parasitoid would probably be much easier if there was no background odor. These findings are consistent with the hypothesis that the reduction of the CHC amount might serve as a second strategy of *H. rutilans* females to evade olfactory detection by beewolf females.

There are only two reports of a dual strategy of chemical mimicry and reduced amount of CHCs. Both deal with social parasitic wasps of the genus *Polistes* (Lorenzi & Bagneres 2002; Lorenzi et al. 2004). In these species, the reduced amount of CHCs facilitates an integration of the parasites into the host colony. Other examples of a low abundance of CHCs as a strategy to escape recognition concern intraspecific parasitism in ponerine ants (Jeral et al. 1997) and interspecific parasitism in slave making ants (D'Ettorre & Errard 1998; Lambardi *et al.* 2007; Lenoir *et al.* 2001).

Compared to studies on the qualitative composition of CHCs, there is a surprising lack of data on the quantity of CHCs on insect cuticles. Those few data available for Hymenoptera are hardly comparable between species, because all authors use different measurements (e.g. µg CHCs/individual after 10 min of hexane extraction in *Vespula germanica* workers (Brown et al. 1991); µg/body surface unit, calculated with the area of the second discoidal cell as a standard for body size after 1 min of pentane extraction in workers of *Polistes dominulus* (Lorenzi et al. 2004); µg/mg of insect after 60 s of hexane extraction in *Polistes annularis* workers (Espelie & Hermann 1990). The new method of a calculation of the amount of CHCs per mm^2 of body surface as presented in this study, would allow a standardized comparison of the amount of CHCs across species.

The CHCs of the beewolves might be transferred to the nest wall by two ways: First, CHCs might be mechanically transferred to the nest wall during the digging activity of the beewolf females. The animals get in contact with the nest wall especially with their legs (tarsal spines of the frontal legs) and the abdomen while processing the excavated sand and also with the head when digging with the mandibles. Second, potentially large amounts of CHCs might be transmitted to the nest walls as a consequence of the unique chemical treatment of the honeybee prey: beewolf females apply large amounts of a secretion from the postpharyngeal gland (PPG) onto their prey by extensive licking prior to egg-laying. This secretion is identical to the CHCs of the beewolf females (Strohm et al. in prep.) and it protects the prey from infestation by fungi (Herzner & Strohm 2007). Treated bees are regularly moved around inside the nest during excavation of the brood cell (Simon-Thomas & Veenendaal 1978) and a part of the applied PPG-secretion might be mechanically transferred to the nest wall. Since beewolf females apply huge amounts of secretions onto the bees, this mechanism might be responsible for most of the hydrocarbons on the nest walls (Herzner & Strohm 2007). Thus,

the treatment of honeybees to prevent fungus growth might be at least one reason why there is a considerable background odor on the nest walls of beewolf nests that facilitates the concealment of the cuckoo wasps both by qualitative and quantitative measures. Notably, other solitary bees and wasps are not known to treat their provisions in this way. Thus, the preconditions for chemical deception in parasites of these host species might considerably differ from the *P. triangulum/H. rutilans* association.

Unlike in social wasp species, there have been no reports about CHCs on the nest material of solitary species. In social species, typical CHC profiles can be recovered from nest material (usually from paper nests) (Dani 2006; Espelie & Hermann 1990; Lorenzi & Caprio 2000; Singer *et al.* 1992; Singer & Espelie 1996; Sumana *et al.* 2005). In these species, the CHCs present on the nest material sometimes even play a role in the context of nestmate recognition. In the case of the European beewolf, the CHCs present on the nest-wall material and also on the nest mounds (Kroiss et al., unpublished data) might also allow individual nest-recognition by the beewolf females, but also facilitates olfactory nest-finding by the cuckoo wasps (Kroiss and Strohm, unpublished data).

The primary function of CHCs is a protection against the evaporation of water, which is especially important in animals with small body size, as it is the case in most insects, and in arid habitats (Gibbs 1998; Lockey 1988). Therefore, the considerable reduction of the CHCs of the cuckoo wasp by a factor of 4.6 compared to its host might impose the problem of water loss and desiccation. This presumptive handicap might, however, be balanced by a dilution of recognition cues for the beewolf host. Thus, *H. rutilans* not only qualitatively mimics the CHC composition of its host (Strohm et al. 2008), but might also employ a quantitative strategy in the sense of a “chemical insignificance”. This is the first evidence of a parasitoid of a solitary aculeate host to use a dual chemical strategy to evade detection.

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CHAPTER 11

A CUTICULAR MULTILAYER GENERATES THE IRIDESCENT COLORATION IN CHRYSIDID WASPS (HYMENOPTERA, CHRYSIDIDAE).

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11.1 SUMMARY

Chrysidid wasps are famous for the spectacular iridescence of the cuticle in some species. They are brood parasitoids or cleptoparasites of other insects and should thus, be rather cryptic than as colorful and conspicuous as they are. However, neither the ultimate causes of this coloration nor the proximate mechanism resulting in this coloration are known so far. In this study, we examine the dorsal abdominal cuticle of the chrysidid wasp *Hedychrum rutilans*. Using Scanning Electron Microscopy, reflectance spectral analysis, and theoretical calculations, we demonstrate the presence of a multilayer consisting of eight slabs with a thickness of 190 nm each in the exocuticle. The slabs exhibit a rough surface probably functioning as spacers between the individual layers. The multilayer reflector has a measured reflectance maximum at λ = 629 nm, i.e. in the red part of the visible spectrum of light at normal incidence and the reflectance maximum shifts to green as the angle of incidence increases. Theoretical calculations using an infinite photonic-crystal model corroborate these results and support the view that the cuticular multilayer generates the iridescent color of the chrysidid cuticle. Our study is the first characterization of cuticular structural colors in the Hymenoptera. Potential ultimate causes for the evolution of the cuticular multilayer in chrysidid wasps are discussed.

11.2 INTRODUCTION

Chrysidid wasps (Hymenoptera, Chrysididae) are a family of small to medium sized obligate parasitoid wasps. All species exhibit a parasitic lifestyle and they are therefore referred to as "cuckoo wasps". Most species show a highly iridescent coloration, except for the Loboscelidiinae and the Allocoeliini, two groups without any iridescence, and some species with partially reduced iridescence (e.g. in the genera *Cleptes*, (Cleptinae), *Parnopes* (Parnopinae), and *Hedychridium* (Elampinae); Kimsey & Bohart 1990). The coloration of Chrysidid wasps has been the subject of investigations for about 70 years (Frey 1936). So far, however, no final conclusion about the proximate mechanism of color generation can bee drawn.

Similarly, the ultimate function of the bright coloration of chrysidids is not resolved and not as obvious as in sexually dimorphic groups like butterflies, where only the males are brightly colored whereas the female shows cryptic coloration (Kinoshita et al. 2002; Vukusic et al. 1999). In these cases, the coloration has evolved in response to sexual selection. In cuckoo wasps, however, both sexes are iridescently colored and sexually dimorphic coloration is only rarely observed. Moreover, since female chrysidid wasps often perch in the vicinity of host nests they should be rather cryptic than brightly colored in order to evade detection by their host.

It is known that the cuticle of cuckoo wasps is unusually hard and sculptured and bears few hairs (Kimsey & Bohart 1990; Kunz 1994). Many species are able to adopt a rolled-up position, thereby protecting the vulnerable legs and the soft ventral side of the abdomen from predators and – probably more importantly – from the hosts.

Hedychrum rutilans is a medium-sized cuckoo wasp and is distributed throughout Central and South Europe. It is a brood parasitoid of the digger wasp genus *Philanthus* (Hymenoptera, Crabronidae). However, since only the European beewolf *Philanthus triangulum* is reasonably abundant in most regions, this is the primary host of *H. rutilans* in most regions. Females of this cuckoo wasp enter the burrow of the subterranean beewolf nests and oviposit onto the prey of the beewolves (Kimsey & Bohart 1990; Kunz 1994; Strohm et al. 2008; Strohm et al. 2001). In the closd brood cell, the cuckoo wasp larva kills the host larva and feeds on the provisions. Coloration of *H. rutilans* males and females does not differ. In both sexes, the abdomen is usually monochromatic red (Fig. 11.1), whereas the thorax and the head are blurred greenish to blue with dispersed reddish and coppery parts. However, the color changes somewhat when the surface is viewed from the side.

Fig. 11.1: Cuckoo wasps (Hymenoptera, Chrysididae) are a family of obligate parasitoids and most species exhibit a strongly iridescent coloration. The abdomen of *Hedychrum rutilans*, a parasitoid of the European beewolf *Philanthus triangulum*, is red at perpendicular view and turns green and blue under larger incidence.

Colors are generated by interactions of light either with pigments or with microstructures, which are in the same order of magnitude as the wavelength of the light (Parker 2000). While pigment colors are widespread in nature, structural colors are relatively rare. Nevertheless, they draw a lot of attention due to their spectacular appearance, often connected with gleam and iridescence. Structural colors can be found for example in butterfly wings (Kinoshita et al. 2002; Vukusic et al. 1999) or bird feathers (Zi et al. 2003), but also in non-living matter like opal (Parker et al. 2003). While it was known for long that these color phenomena have a physical rather than a pigmentary origin, the exact mechanism of color origin especially in insects was not understood for a long time. The discovery of photonic crystals (John 1987; Yablonovitch 1987) has strongly inspired the research on biological photonic crystals and one- to three-dimensional photonic crystals have been discovered to be the cause of structural colors in nature (Kinoshita & Yoshioka 2005). These investigations were mostly on butterfly wings (Biro 2007; Ghiradella 1991; Ghiradella 1994; Kinoshita et al. 2002), but also on beetles (Parker et al. 2003; Vigneron et al. 2006; Welch et al. 2007), and bird feathers (Doucet et al. 2006; Zi et al. 2003).

In this study, we investigate the proximate causes of iridescent coloration of the cuticle in the cuckoo wasp *H. rutilans* using Scanning Electron Microscopy (SEM) and reflectance spectral

analysis and compare the results with theoretical calculations. We additionally discuss the potential ultimate causes for structural colors in cuckoo wasps.

11. 3 MATERIALS AND METHODS

11.3.1 Scanning Electron Microscopy

Females of *Hedychrum rutilans* (Hymenoptera: Chrysididae, Chrysidinae) were caught near Nürnberg, Germany. The wasps were freeze-killed and later thawed and air-dried for several days prior to preparation. Parts of the air-dried dorsal abdominal cuticle were obtained by use of freeze-fracture technique (Vigneron et al. 2006). The broken cuticle was sputtered with gold (15 nm) and examined through a Phillips XL20 SEM.

11.3.2 Reflectance spectral analysis

For the reflectance spectral analysis, three abdomens of *H. rutilans* were ventrally glued (dorsal side up) onto a glass slide covered with black construction paper using superglue. Since the abdomens were slightly curved, they were flattened by compression. An Avaspec 2048/2 fiber optic spectrometer was used for reflectance spectral analysis under 0°, 15°, 30°, 45°, 60°, and 75° incidence in a specular geometry with wavelengths ranging from 250 to 1000 nm. The intensity reflected is expressed in units of the corresponding diffuse reflection obtained on a standard white (Avaspec) diffusing surface. This method constitutes a common way of normalization and leads to the expression of a “reflection factor”, which is not necessarily delimited by 100%.

11.3.3 Calculation of the dominant reflected wavelength

Instead of using detailed Fresnel formulae for the calculation of the dominant reflected wavelength of a finite stack of layers, we used an approach based on an infinite multilayer with strict periodicity, i.e. a one-dimensional photonic crystal, as described by Vigneron et al. (2006). This approach uses the Bloch theorem and has been shown to be suitable for

photonic structures (multilayers) of biological origin (Vigneron et al. 2006). The dominant reflected wavelength λ of a multilayer along the z direction can be calculated using the formula

$$\lambda = \frac{2a\sqrt{\bar{n}^{-2} - \sin^2 \Theta}}{m} \qquad (1)$$

with a being lattice constant, $\bar{n}$ being the average refractive index of the multilayer, Θ being the angle from normal along z, and m being an integer (m = 1, 2, 3, ...). For a detailed derivation of this formula see Vigneron et al. (2006). At these frequencies, total reflectance will occur and coloration of the multilayer is determined by this wavelength. Since sin (0) = 0, the reflected wavelengths for Θ = 0° are

$$\lambda = \frac{2a\bar{n}}{m}. \qquad (2)$$

The refractive index of pure chitin is n_c = 1.56 (Land 1972; Vukusic et al. 1999). Due to the inclusion of other substances into the chitinous matrix, the refractive index (RI) of insect cuticle can reach values as high as n_c = 1.68 (Noyes et al. 2007). For the calculations of the dominant reflected wavelength, we initially used a RI of n_c = 1.56. However, we found a slight mismatch between the measured and the calculated reflectance maximum. Therefore, we adjusted the RI of the cuticular multilayer to $\bar{n} = 1.64$, which is well within the range of reported RIs for cuticular material (see above).

11. 4 RESULTS

11.4.1 Scanning Electron Microscopy

Scanning electron microscopy of the fractured cuticle revealed a total thickness of the dorsal abdominal cuticle of about d ≈ 27 µm. The cuticle exhibits a roughly layered structure with a layer thickness increasing from 550 to 1000 nm from the inner to the outer side of the cuticle. On the outermost part of the cuticle, the exocuticle, there was a distinct multilayer with a total thickness of m ≈ 1.54 µm (Fig. 11.2). This multilayer consists of about eight slabs of a chitinous material with a lattice constant of a ≈ 190 nm (Fig. 11.3). Scanning electron

micrographs of angular broken cuticle revealed a terraced layering of the slabs, suggesting that the multilayer consists of a simple planar multilayer with chitinous layers of equal thickness (Fig. 11.4). The surface of the layers is irregularly rough. This roughness potentially serves as a spacer between the individual layers.

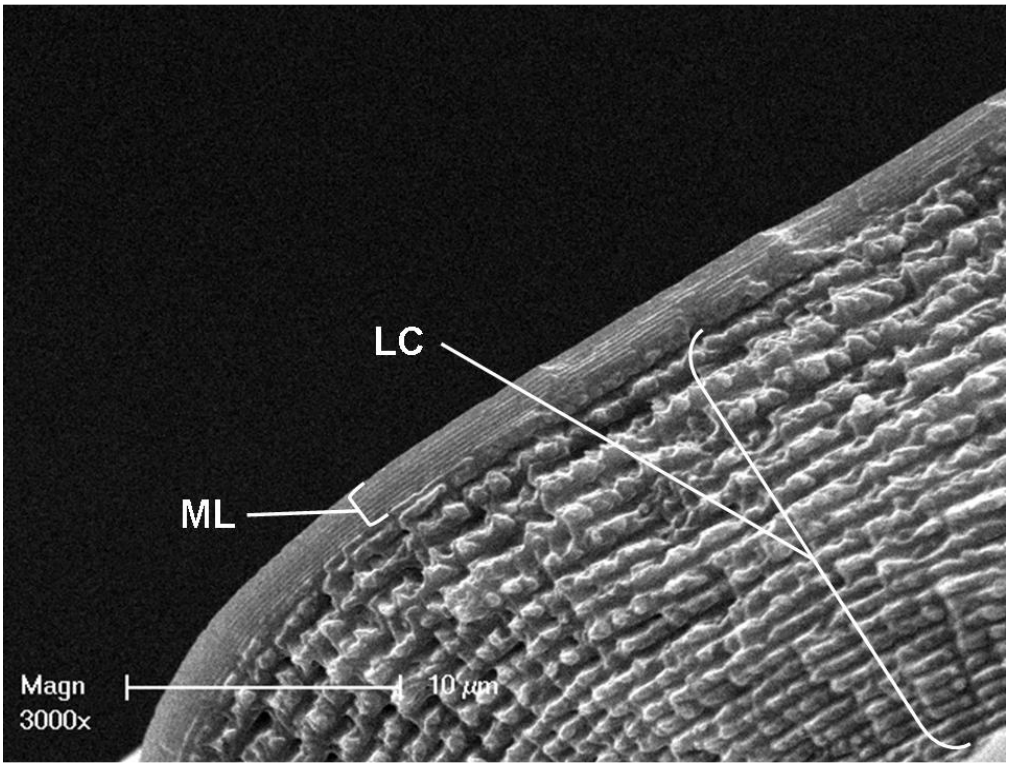

Fig. 11.2: Scanning electron micrograph of the broken dorsal abdominal cuticle of *H. rutilans*. The cuticle has a total thickness of d ≈ 27 µm. The multilayer reflector is visible on the outermost side of the cuticle with a total thickness of m ≈ 1.54 µm (ML). Below the multilayer stack lays the roughly layered endocuticle with the layer thickness ranging from 1000 to 550 nm moving from the outward side to the inner side of the cuticle (LC).

11.4.2 Reflectance spectral analysis

The reflectance spectral analysis in the spectrum between 250 and 1000 nm revealed one dominant reflectance maximum at λ ≈ 629 nm at normal incidence (Fig. 11.5). This wavelength corresponds to the red to orange part of the human visible spectrum. The wavelength of maximum reflectance stays rather constant for angles of incidence of 15° (630 nm) and 30° (629 nm). As the angle of incidence increases further, the dominant reflectance maximum switches to 562 nm at 45° (yellow to green), 536 nm at 60° (green), and 510 nm at 75° (green) (Fig. 11.5). These measurements are in congruence with the blue-shift observed with the naked eye: the color of the abdominal cuticle changes from red to green as the angle of view increases.

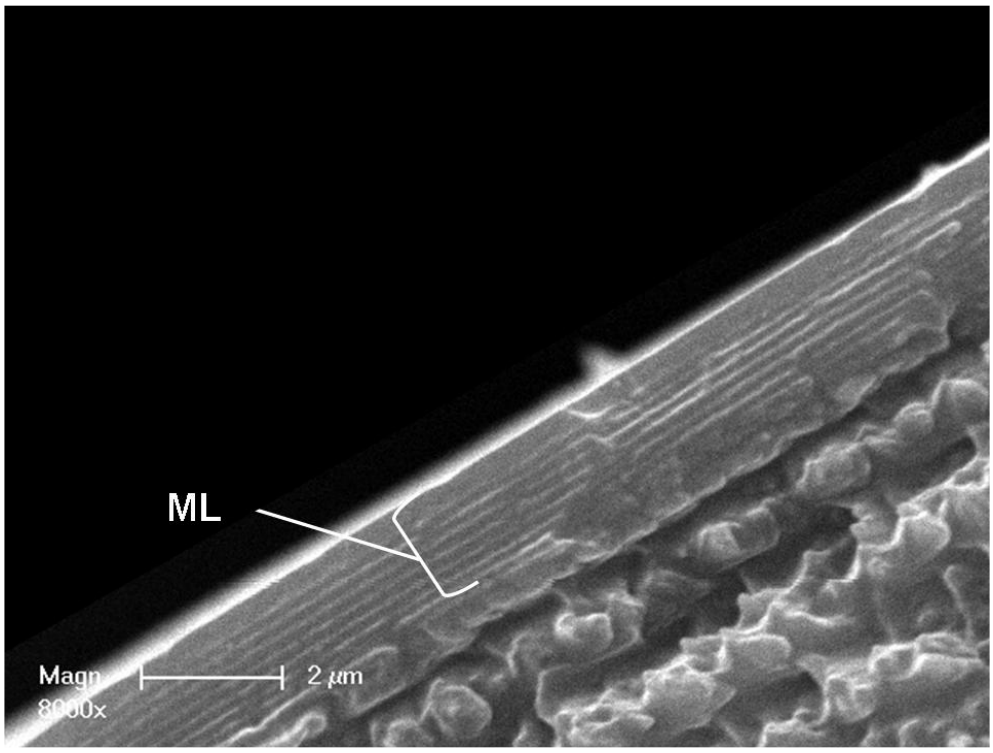

Fig. 11.3: Scanning electron micrograph of the cuticular multilayer reflector (ML) of *H. rutilans*. The thin layer on the surface of the dorsal abdominal cuticle of *H. rutilans* is a stack of about 8 slabs of a chitinous material with a lattice constant a ≈ 190 nm.

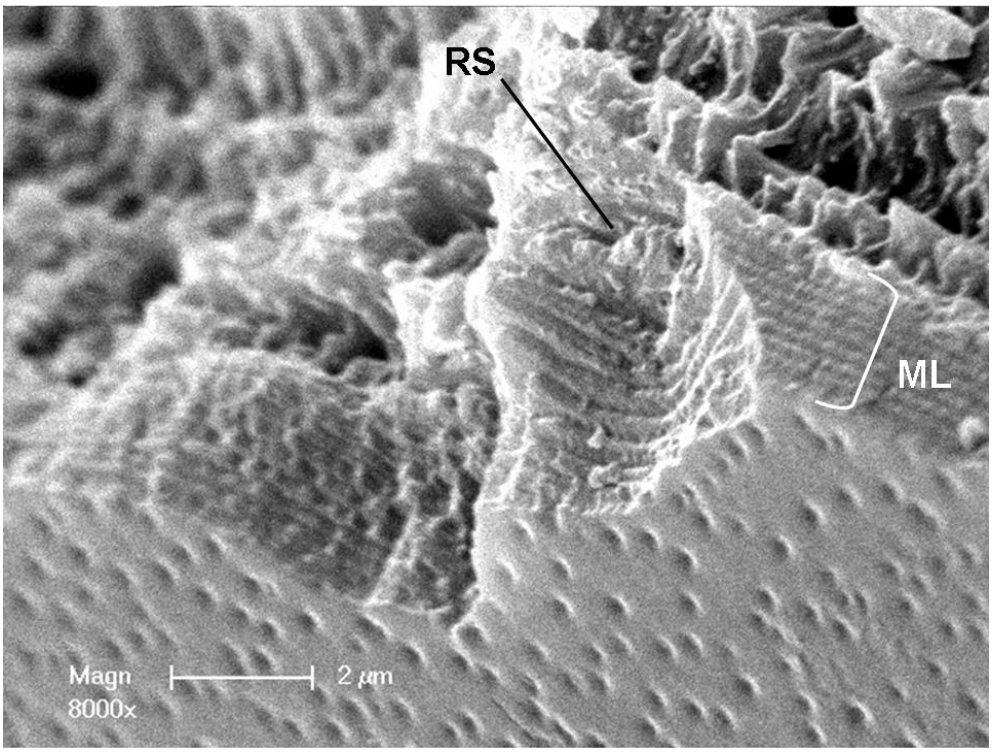

Fig. 11.4: Scanning electron micrograph of the cuticular multilayer reflector (ML) of *H. rutilans*. The cuticle is broken in an angular manner and the surface of some of the chitinous slabs is visible in the terraced area. On the surface of the slabs, some irregular roughness can be observed (RS), which potentially serves as a spacer element between the slabs.

Besides the dominant reflected wavelength, the reflectance spectral analysis revealed several other reflectance maxima (Fig. 11.5). At normal incidence, the wavelengths of these maxima were 315 nm, 430 nm, and 755 nm, respectively. As with the dominant reflectance maximum, these maxima also showed a blue-shift with increasing incidence of the light.

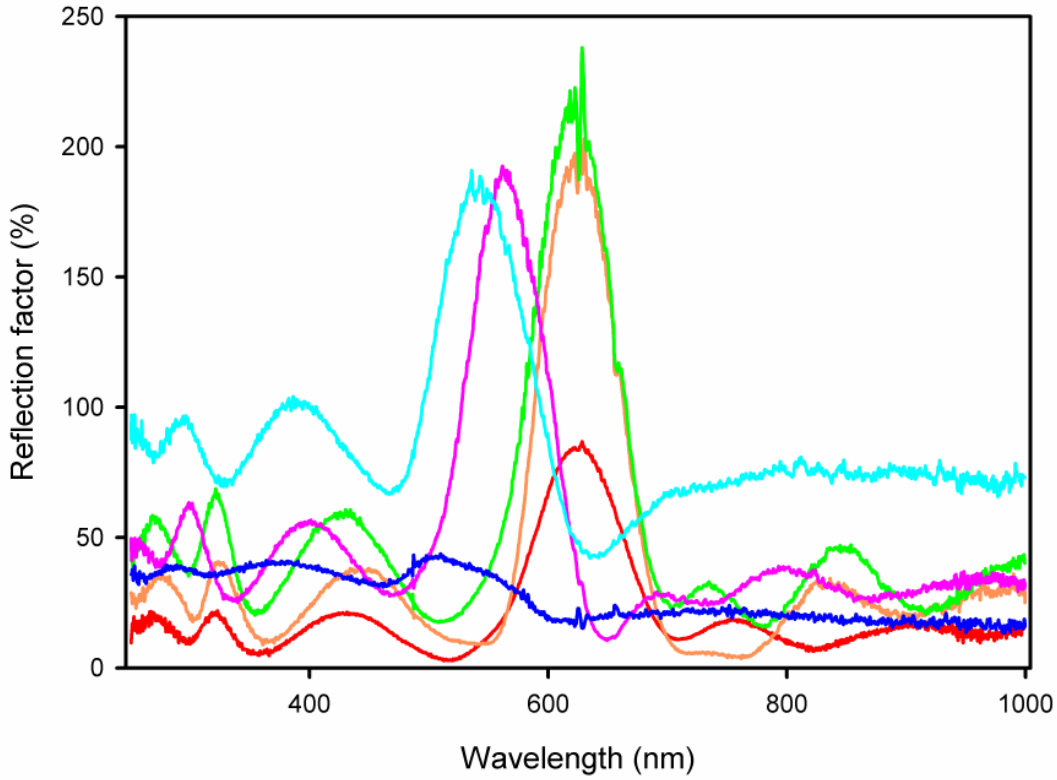

Fig. 11.5: Spectrum of light reflected from the abdominal dorsal cuticle of *H. rutilans* for six angles of incidence (red line: 0°, orange line: 15°, green line: 30°, magenta: 45°, turquoise: 60°, blue: 75°). The band of maximum reflection shifts from red to green as the angle of incidence increases. The intensity of reflected light is expressed in % compared to a standard white reflector (see text for details).

11.4.3 Calculation of the dominant reflected wavelength

The calculation of the dominant reflected wavelength of the cuticular multilayer (formula (2)) revealed a dominant reflectance band for $\Theta = 0°$ and $m = 1$ at 623 nm. With an increasing angle of incidence (formula (1)) the dominant reflected wavelength for $m = 1$ shifted to 562 nm for $\Theta = 45°$ and 503 nm for $\Theta = 75°$. The second reflected wavelength for $\Theta = 0°$ and $m = 2$ is at 311 nm.

11. 5 DISCUSSION

The SEM micrographs revealed the existence of a multilayer in the dorsal exocuticle of the abdomen of *H. rutilans*. We found eight layers with a thickness of about 190 nm each. The surface of the single layers is rough and this roughness might serve as a spacer between the layers (Vigneron et al. 2006). However, the nature of the material between the individual layers is not known. It is possible and plausible that the space between the layers is – apart from the chitinous spacers – filled with air. Alternatively, it might be another material with a refractive index differing from that of the chitinous layers themselves. With the SEM micrographs of our study, it is not possible to discriminate between these two alternatives, though it seems more likely that the space between the layers is filled with air. However, it is important to note that for the modeling of the dominant reflected wavelength it makes no difference, which material is present in the gaps between the single layers, since only the average refractive index $\bar{n}$ is required for the model (Vigneron et al. 2006). As the distance between the single layers is very small, it contributes only very little to the average refractive index. The observed multilayer has a rather simple construction and all layers have the same thickness. A fairly similar multilayer has been discovered in buprestid beetles (Vigneron et al. 2006), however, the thickness of the layers as well as the number of layers are larger than in the cuckoo wasp.

The reflectance spectral analysis revealed one reflectance maximum at λ = 629 nm at normal incidence, which is in the red to orange part of the visible spectrum of light. This measurement is consistent with the calculated dominant reflected wavelength (λ = 623 nm at Θ = 0° and m = 1). As expected for a multilayer reflector, we observed a clear blue-shift with increasing angle of incidence from red to green. Likewise, such a blue-shift was predicted by the calculations, and the calculated and measured values are also in good congruence. Apart from the predominant reflectance maximum in the red part of the visible spectrum, we observed three other reflectance maxima in the spectral range from 250 to 1000 nm. The reflectance maximum at λ = 315 nm can easily be interpreted as the second reflectance maximum of the cuticular multilayer reflector (m = 2). The causes for the other reflectance maxima at λ = 430 nm, and λ = 755 nm, however, are not yet clear.

Below the cuticular multilayer, the endocuticle exhibits regular layers with the layer thickness ranging between 550 and 1000 nm. Potentially, these layers represent reflectors of light in the infrared range. The potential main reflectance maximum for m = 1 is not visible in our reflectance spectral analysis, because the analysis was restricted to the wavelengths ranging from 250 to 1000 nm. The observed reflectance maxima might constitute reflectance maxima

of the roughly layered endocuticle for m > 1. However, this hypothesis remains to be tested by recording reflectance spectra for the infrared range.

Our study clearly shows the existence of a cuticular multilayer. The physical properties of this multilayer suggest that it is responsible for the generation of the iridescent coloration of the cuckoo wasp *H. rutilans*. The ultimate causes for the coloration are not as clear. Though intraspecific communication can not be ruled out as an ultimate function of the iridescent coloration of cuckoo wasps, it is possible that the coloration primarily evolved in response to other demands. First, the coloration might represent an aposematic signal to deter predators. However, the sting of cuckoo wasps is not an effective weapon, thus they could only make use of Batesian mimicry, but a potential model is not known. Second, the coloration might signal indigestibility. There is no evidence available that might support or weaken this alternative.

Third, the coloration might just be an epiphenomenon of the structure of the cuticle that was selected for other properties. Consistent with this assumption, the cuticular multilayer might enhance the mechanical strength of the cuticle. The multilayer might function like spaced armor (Brown & Turner 1985) and might protect the cuckoo wasps from attacks of their hosts with the sting or the mandibles. The fact that in *H. rutilans* iridescent coloration can only be seen on body parts exposed when adopting the rolled-up defensive posture supports this hypothesis. Furthermore, the cuticular multilayer – potentially in combination with the roughly layered endocuticle – might allow a better thermoregulation in the usually hot and dry habitats of the cuckoo wasps. Structural colors have been shown to reduce heating by solar irradiation (Biro et al. 2003) and might therefore facilitate the cuckoo wasps to stay longer in the usually warm and dry surrounding of the nests of their hosts. Nevertheless, it is also possible that the photonic structures have evolved in response to several selective pressures (Welch & Vigneron 2007). The structures might be a compromise solution in that they are well suited to serve some of the selective demands (structural rigidity, reflectance), but might be less optimal for others (detection by hosts).

In conclusion, our analysis provides convincing evidence that a cuticular multilayer generates the iridescent coloration of the cuckoo wasp *H. rutilans*. Though the ultimate reason for the structural colors is not yet clear, this is the first description of a cuticular one-dimensional photonic crystal in the Hymenoptera.

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CHAPTER 12

GENERAL DISCUSSION

12.1 THE BEEWOLF SEX PHEROMONE AND ITS POTENTIAL FOR FEMALE MATE CHOICE

In the first part of this thesis, we describe the composition and site of production of the male sex-pheromone of European beewolves. We show that extraordinarily large amounts of the pheromone are stored in a postpharyngeal gland (PPG) and we describe the composition of the pheromone in detail (chapters 2 and 3). The fact that huge amounts of the pheromone are produced by beewolf males suggests that the large quantity of the pheromone might be the result of strong sexual selection and might allow female choice according to a good genes model. Furthermore, we demonstrate that the multi-component pheromone may provide information about the geographical origin and kinship of the males and might allow female choice according to a best compatibility model (chapter 4). Specific issues of these aspects have been discussed in the particular chapters. Therefore, in this paragraph I will discuss general aspects of the evolution of the beewolf male sex pheromone and female choice according to two models of genetic benefits, i.e. the model of good genes and the model of good compatibility and I will integrate knowledge from the beewolf system into information from other taxa. Furthermore, I will consider theoretical problems arising from the good genes model in combination with theoretical problems connected with the lek mating system of European beewolves described in this thesis (chapter 5) and peculiarities of the beewolf biology.

Due to the asymmetry between the sexes, females are in general expected to be choosier than males with regard to mate choice (Andersson 1994; Gould & Gould 1997; Trivers 1972). Females might choose their mates according to direct (Halliday 1983; Hamilton & Zuk 1982; Vahed 1998) or indirect benefits (Andersson 1994; Møller & Alatalo 1999; Wilkinson et al. 1998). Beewolf males do not provide any resources to females or provide any brood care. Therefore, beewolf females are expected to benefit mostly indirectly, i.e. genetically from adaptive mate choice.

12.1.1 The beewolf sex pheromone as a possible exaggerated chemical signal and its potential as an honest indicator of good genes

Our reanalysis of the male sex-pheromone of European beewolves with the pheromone directly recovered from the PPG (chapter 2) confirmed that male beewolves possess a multi-component sex-pheromone with *(Z)*-11-eicosen-1-ol as the main substance (see also Schmidt et al. 1990; see also Schmitt et al. 2003). We were able to identify 42 more substances than in earlier analyses. Apart from several substances with functional groups, we predominantly found linear and branched alkanes and alkenes in the PPG. Recent analyses reveal that not only eicosenol, but also the other substances with functional groups as well as the hydrocarbons can be found in extracts of marked territories and in the headspace of these territories (J. Kroiss et al., unpubl. data). Therefore it is evident that all the substances found in the PPG of male beewolves are part of the pheromone and are in principle perceivable to females. However, so far it has not been elucidated, which of these substances are actually perceived by females and whether the individual substances have different functions e.g. for species recognition, remote mate attraction or mate assessment and individual mate choice. Electrophysiological investigations (GC-EAD) would allow identifying substances that are in principle physiologically perceivable by beewolf females. Bioassays with single substances or a combination of several compounds might help to assign specific functions to particular pheromone compounds.

Nonetheless, the extraordinarily large amounts of the pheromone stored by beewolf males in the huge PPG and its great variability (mean ± SD = 653 ± 377 µg, range: 101 – 2508 µg, n = 393; see chapter 4) suggests that the production of the pheromone may be condition-dependent and that the amount of pheromone produced may play a crucial role for the reproductive success of males. The amount of pheromone applied onto the territory substrate might function as an honest indicator of male quality. In this case, only particularly good males might be able to “afford” the production of large amounts of pheromone and would therefore have a high probability of attracting receptive females. In principle, the multi-component pheromone as a whole or single substances – especially eicosenol – might play a role for mate choice according to quantitative aspects of the pheromone. Large amounts of the pheromone might additionally allow female attraction over a long distance. It would be interesting to test for a condition-dependence of the pheromone production in beewolf males. Males can easily be kept under restricted food supply and starved males may not be able to afford the production of large pheromone amounts. Female choice experiments with differently nourished males might reveal a choice of males that produce large amounts of pheromone.

Though several pieces of the mosaic of the evolution of the male sex-pheromone in beewolves are resolved now, the complete picture of the origins and the subsequent evolution of the pheromone are still not completely clear. There is now considerable evidence that the presence of eicosenol in the pheromone is the result of exploitation by the males of a preexisting sensitivity of females for this substance that originated in the context of prey hunting according to the sensory exploitation model (Herzner 2004; Herzner et al. 2005; Schmitt et al. 2007). Subsequently, males might have been strongly selected for the production of large amounts of eicosenol.

Two models have the potential to explain the extraordinarily large amounts of the pheromone in general and specific substances – especially eicosenol – in particular. Following the runaway model of R. Fisher (1930), females will choose their mates according to an arbitrary criterion, and therefore, the criterion itself and the preference will be co-inherited ("Fisher's runaway" or "sexy son" hypothesis). Transferred onto the beewolf system, the criterion of female choice would be of course not completely arbitrary, but the result of sensory exploitation. In any case, the coinheritance of the male trait and the preference will lead to the evolution of exaggerated male traits and the preference of females for this trait.

One can alternatively assume that the amount of the beewolf pheromone is – following the exploitation of the females' sensitivity – not the result of a runaway selection process in the sense of Fisher (1930), but the result of female selection for an honest signal of male quality in the narrow sense of a good genes model. The large amounts of the pheromone might provide the female with reliable information about the general physiological and metabolic abilities of the males. Beyond that, the pheromone might convey information about specific physiological capabilities of the males: as pointed out before, several long-chained hydrocarbons are part of the male pheromone. Interestingly, these substances are also located on the male (see chapter 3) and the female cuticle (see chapter 8), but more importantly, they are also the main substances to be found in the PPG secretion of beewolf females (Strohm et al. 2008; Strohm et al. in prep). This secretion, a hydrocarbon mixture with alkenes as the main components, is applied by the beewolf females onto the honeybees that serve as food for the beewolf larvae. The secretion serves to avoid molding of the honeybee prey in the warm and humid underground nest (Herzner et al. 2007; Herzner & Strohm 2007; Herzner & Strohm accepted). Fungal and bacterial decay of the honeybees is a serious threat to the developing larvae (Strohm & Linsenmair 2001) and treatment of the stored honeybees is an important fitness component for females. From the presence of these hydrocarbons in the pheromone, females may gain specific information about the males' abilities to synthesize these crucial hydrocarbons. Females will potentially benefit from mating with a male with a good ability to synthesize the hydrocarbons involved in bee

treatment. The physiological abilities of the males with regard to the synthesis of long-chained hydrocarbons might be of special interest for the females, because due to haplodiploidy the male genes are only transmitted to and expressed in daughters.

As a summary, the total amount of the pheromone and the amount of specific pheromone compounds might convey information about general and specific physiological and metabolic characteristics of the males. The amount of the pheromone is equivalent to the intensity of other sexual signals like the volume of bird song (Brown 1999) or the intensity of courtship display (Cubillos & Guderley 2000; Doty & Welch 2001).

Apart from metabolic capacities, several other factors have been discussed as honest indicators of male quality, among them size (Cooper Jr & Vitt 1993; Kotiaho et al. 1996) and age (Beck & Powell 2000; Brooks & Kemp 2001; Hansen & Price 1995; Kokko 1997; Kokko 1998; Kokko & Lindstrom 1996; Manning 1985; Proulx et al. 2002, but see Howard et al. 1994; Hunt et al. 2004, see also Osada et al. 2003; Vainikka et al. 2006 for age-related changes in pheromones). The beewolf pheromone has been demonstrated to contain information about both of these factors (Kaltenpoth 2006; Kaltenpoth & Strohm 2006). Therefore, the beewolf pheromone has the potential to convey extensive information about possible quality indicators of a male and might allow adaptive female choice according to these pheromone characters in the sense of the good genes model, making the beewolf pheromone a “chemical peacock tail”.

Fisher’s runaway theory and the model of good genes might represent two points of a continuum (Kirkpatrick & Ryan 1991; Kokko et al. 2002; Mead & Arnold 2004; but see Andersson & Simmons 2006; Cameron et al. 2003). In both cases, the preference of the female becomes correlated with the male trait when both have a genetic basis (Bakker & Pomiankowski 1995; Houde & Endler 1990; Wilkinson & Reillo 1994). In both models females choosing a mate by means of the exaggerated ornaments will leave descendants with a high fitness (Houle & Kondrashov 2002; Pape Moller 1994).

Adaptive female mate choice according to the good genes model and Fisher’s runaway model has been demonstrated in several species. In particular, it has been shown that mate choice by females can have positive fitness consequences (Barber et al. 2001; Doty & Welch 2001; Partridge 1980; Promislow et al. 1998; Sauer et al. 1998; Sheldon et al. 1997; Tallamy et al. 2002; Wilkinson & Reillo 1994). Furthermore, a genetic correlation between male trait and female preference has been shown in several studies (Andersson 1994; Andersson & Simmons 2006; Bakker 1993; Bakker & Pomiankowski 1995; Pomiankowski 1988; Wilkinson & Reillo 1994). The focus of these studies, however, is mostly on species where males

advertise their quality using visual or acoustic signals. Several studies have addressed the potential of chemical cues to convey honest information about male quality in insects (Beeler et al. 2002; Droney & Hock 1998; Kortet & Hedrick 2005; Moore 1997; Thornhill 1992), fish (Reusch et al. 2001), amphibians (Marco et al. 1998), reptiles (Martin & Lopez 2000) (see also Lemaster & Mason 2002), and mammals (Kavaliers et al. 2003; Willis & Poulin 2000). Only few studies have shown adaptive mate choice according to chemical signals (Andersson et al. 2007; Jones et al. 2000; Jones & Hamilton 1998; Jones et al. 1998; Vainikka et al. 2006).

12.1.2 Problems of good genes theory – sexual conflict and sexual antagonism

Although the good genes theory has the potential to explain the exaggerated male ornaments puzzling Charles Darwin (1859), there are some restrictions of this theory that have to be taken into account. One such limitation of the good genes theory is the sometimes observed negative correlation between male and female fitness, meaning that "good" males will have "bad" daughters and "good" females will leave "bad" male descendants. Such a phenomenon of sexually antagonistic genes (SAGs) has been shown e.g. for *Drosophila* (Arnqvist & Rowe 2002; Chippindale et al. 2001; Gibson et al. 2002; Oneal et al. 2007; Pischedda & Chippindale 2006), a species with a XX/XY chromosomal sex-determination mechanism. In such species, many of the sexually selected genes are located on the X-chromosome and males only transmit a Y-chromosome to their sons. Thereby, high fitness can not be passed on from a male to his sons. Sexual selection is interrupted in species with a XX/XY sex-determination mechanism and females should choose their mates according to genes beneficial for their daughters (Albert & Otto 2005).

In species with a ZW/ZZ sex-determination mechanism like birds with the males being the homogametic sex, the situation is changed: males transmit one of their Z-chromosomes, which bears a great proportion of the sexually selected genes, onto their sons. Sexual selection is not interrupted in such species and females should prefer males with genes beneficial for their sons. Taxa with a ZW/ZZ sex-determination mechanism frequently exhibit exaggerated male sexual signals.

The findings from XX/XY and ZW/ZZ systems can be transferred onto the beewolf system only to a limited degree. However, the haplo-diploid sex determination of the Hymenoptera has a greater similarity to the XX/XY-system (Margolies & Cox 1992). Males of haplo-diploid organisms pass on their genes exclusively to daughters, corresponding to the X-chromosome

in *Drosophila*, which will therefore lead to an interruption of sexual selection. As a result, females should choose males carrying good genes for their daughters (Albert & Otto 2005).

In conclusion, beewolf females might in fact choose their mates according to quantitative aspects of the male pheromone, thereby getting honest information about the ability of the male to produce the hydrocarbons involved in prey conservation in beewolf females (i.e. genes important for female offspring). Although sexual selection is interrupted in haplo-diploid species, choosing an extraordinarily good male for mating might additionally lead to an especially good grandson of the choosing female ("sexy grandson").

Sexual conflict, however, might be expected for other genes like the ones involved in the biosynthesis of eicosenol or the other substances with functional groups. Although some biosynthetic pathways of alkanes and alkenes as well as substances with functional groups are similar, specific enzymes are needed for each compound (Choi et al. 2005; Choi et al. 2002; Howard & Blomquist 2005; Tillman et al. 1999). Additionally, sexual conflict might be expected with regard to basal characteristics like longevity. While males might invest heavily into sexual signals early in their lives thereby having a shorter life expectancy (Hunt et al. 2004), females might be selected for longevity, since this might enhance their reproductive success (E. Strohm, unpubl. data).

Apart from SAGs, the maintenance of genetic variability is a further problem of the good genes theory. Since females prefer to mate with the best male available, there is a strong mating skew in a given population. Such a mating skew might be even more pronounced in species with a lek mating system, when males cluster in an arena and advertise their quality with visual, acoustic, or chemical signals (Choe & Crespi 1997). As already discussed in chapter 5, one would assume that genetic variability would be eliminated very quickly in such systems. The fact that there is still a high genetic variability in lek-mating species has become famous as the "lek-paradox" (Kirkpatrick & Ryan 1991; Taylor & Williams 1982; Tomkins et al. 2004). The lek mating system as observed in the European beewolf (chapter 5) and female mate choice according to a good genes model would lead to a loss of genetic variability. However, several factors are expected to contribute to the maintenance of genetic variation in beewolves: different populations obviously differ with regard to their abiotic and biotic environmental factors. Important abiotic factors might be temperature and humidity, biotic factors might be above all parasites (Kaltz & Shykoff 1998), which are critically important for the reproductive success of beewolves (Strohm et al. 2001; Strohm & Linsenmair 2001). Biotic interactions are not constant and an adaptation optimal in the presence may not be optimal in the future. The data presented in chapters 7 -10 indicate an ongoing evolutionary arms race of European beewolves and their major brood parasitoid, the cuckoo wasp *H.*

rutilans. Furthermore, an exchange of individuals between populations, as expected for highly mobile species as beewolves, might sustain genetic variability. The fact that several environmental factors (temperature, humidity, amount of provisions) during the larval development of beewolf males have an influence on pheromone characteristics (K. Roeser-Mueller et al., unpubl. data) and the generally high variability in the pheromone amount produced suggest that the expression of the beewolf pheromone is condition dependent. Such a condition-dependence (Johansson et al. 2005; Kemp & Rutowski 2007; Rantala et al. 2003; Scheuber et al. 2003; Scheuber et al. 2003; Scheuber et al. 2004; Tomkins et al. 2004) would additionally contribute to the maintenance of genetic variation. Furthermore, alternative reproductive strategies (see chapter 5) like satellites apart from territorial males would maintain genetic variation. The interruption of sexual selection due to haplodiploidy, as outlined before, will ultimately also maintain genetic variation. If beewolf females choose their mate not only according to a good genes model but also with regard to the complementarity (chapter 4; see also paragraph 12.1.3), this would also conserve genetic variability to the population.

In conclusion, beewolf females might choose their mates either according to quantitative or qualitative aspects of the male pheromone, territory ownership and position within the lek, or both. Most problems of the good genes theory found in other organisms do not fully apply to the beewolf system due to the haplodiploidy and the specific peculiarities of male and female behavior. Therefore, the strength of sexual conflict is expected to be not exceptionally high in the European beewolf.

12.1.3 The beewolf sex pheromone as an indicator of kinship and its potential as an indicator for "optimal outbreeding"

Sexual signals do not only serve for individual mate assessment, but prior to this also for the identification of the own species, the sex of the emitter, and the reproductive status (Johansson & Jones 2007). Different species are expected to vary with regard to their sexual signals to avoid mating with the wrong species (i.e. pre-mating reproductive isolation) and to minimize the costs of mate searching. This is impressively evident if one listens to the great variety of bird songs. Such a species-specific variation is also known from insect pheromones (Coyne & Orr 1997; Frey et al. 1998; Johansson & Jones 2007; Roelofs 1995; Shine et al. 2002; Symonds & Elgar 2004; Symonds & Elgar 2004; see also Sappington & Taylor 1990). In beewolves, male sex pheromones vary greatly between species (McDaniel et al. 1987;

McDaniel et al. 1992; Schmidt et al. 1990; M. Kaltenpoth, G. Herzner, K. Roeser-Mueller, E. Strohm, unpubl. data) and this variability allows sympatric species to avoid mating with the wrong species.

In the European beewolf, the substances present in the pheromone are rather constant over the whole range of distribution (see chapter 4, M. Kaltenpoth et al., unpubl. data). However, there is some quantitative variability, which allows discriminating between individuals of different populations, subpopulations, and families (see also Herzner et al. 2006). These findings, together with the fact that we found a strong correlation between the geographical distance between populations and their chemical difference, support the view that genetic differences add up with increasing distance according to the model of "isolation by distance" (Wright 1943). The differences in the pheromone composition might also be the result of adaptations to different environments or the result of differing female preferences between populations. In our study, the individuals of different populations were reared under identical conditions to exclude the influence of environmental factors on the pheromone (K. Roeser-Mueller et al., unpubl. data). This suggests that the variability of the beewolf sex-pheromone has a strong genetic component (see also K. Roeser-Mueller, M. Kaltenpoth, G. Herzer, unpubl. data). However, as mentioned before, some environmental factors have a noticeable influence on pheromone characteristics. Yet, despite of different rearing conditions it is still possible to assign family affiliation to individual males (K. Roeser-Mueller et al., unpubl. data). Therefore, the male sex-pheromone of beewolves bears a robust signal about kinship and geographical origin and might allow mate choice according to a model of compatibility/complementarity and optimal outbreeding. Mate choice experiments with males and females from different populations might reveal, whether beewolf females indeed choose their mate according to an optimal outbreeding model.

There is now considerable knowledge about geographical variation of sexual signals like bird song (Kleeman & Gilardi 2005; Nelson & Soha 2004; Packert & Martens 2004; Westcott & Kroon 2002), but also for sex-pheromones. Most studies, however, focus on female sex-pheromones (Gemeno et al. 2000; Grosman et al. 1997; Huang et al. 1998; Kawazu et al. 2000; McElfresh & Millar 1999; McElfresh & Millar 2001; Takanashi et al. 2005; Vereecken et al. 2007; Yatsynin et al. 1996) and knowledge about geographical variation of male sex-pheromones is limited (Aldrich et al. 1989; Hamilton et al. 2004; Hamilton et al. 2005; Ryan et al. 1995; Watts et al. 2005). Geographical variation of sexual signals might allow both the avoidance of in- and outbreeding and mate choice according an optimal outbreeding model (Bateson 1978; Bateson 1980; Bateson 1983). Inbreeding (Akimoto 2006; Aspi 2000; Demeester 1993; Marshall & Spalton 2000), especially in Hymenoptera (Gerloff & Schmid-Hempel 2005; Henter 2003; Zayed & Packer 2005), and outbreeding (Aspi 2000; Brown

1991; Demeester 1993; Marr et al. 2002; Marshall & Spalton 2000; Peer & Taborskyi 2005) have both certain disadvantages and inbreeding avoidance (Foster 1992; Gerlach & Lysiak 2006; Ode et al. 1995; Pusey & Wolf 1996; Pusey 1987; see Vereecken et al. 2007 for the preference of allopatric females by males of *Colletes cunicularius* by means of pheromone differences) and outbreeding avoidance (Harmsen & McKay 1985) has been shown in several taxa (but see Keller & Fournier 2002). Studies of mate choice in the framework of optimal outbreeding have so far not been conducted (but see Palmer & Edmands 2000).

12.2 THE MALE PHEROMONE OF THE CUCKOO WASP *HEDYCHRUM RUTILANS*

In chapter 6 of this thesis, we describe a male-specific substance produced in sternal glands of males of the cuckoo wasp *Hedychrum rutilans*. Though not definitely shown by behavioral assays, this substance might function as a sex-pheromone to attract females for mating. In the following, we discuss the evolution of the sternal glands and the putative sex-pheromone in *H. rutilans*.

Chemically very diverse substances from a plethora of glands have been recently described as male sex-pheromones for a range of different insect taxa (Andersson et al. 2007; Haberer et al. 2007; Johansson & Jones 2007; Kock et al. 2007; Ruther et al. 2007). Sternal glands have been described for male polistine wasps as glands producing male sex-pheromones (Downing et al. 1985; Landolt & Akre 1979; Litte 1979; Post & Jeanne 1982; Post & Jeanne 1983; Post & Jeanne 1983; Turillazzi & Cervo 1982). However, most studies on sternal glands in polistine wasps deal with females. These glands have been reported to either produce an ant-repellent (Dani et al. 1996; Dani et al. 2003; Jeanne et al. 1983; Jeanne 1970; Keeping 1990; Post et al. 1984; Richards 1971; Richards 1978) or a trail-pheromone in the context of swarming behavior during nest founding (Clarke et al. 1999; Jeanne et al. 1983; Jeanne & Post 1982; Smith et al. 2002; van der Vecht 1968; West-Eberhard 1982), depending on the mode of nest founding of the respective species. Many different kinds of sternal glands can be found in ants, where they mostly serve to produce trail pheromones (Hölldobler & Wilson 1990 and references therein). Sternal glands in ants are very diverse and are assumed to have evolved several times independently (Hölldobler & Wilson 1990).

Surprisingly, sternal glands have now been found in males but not in females of the cuckoo wasp *H. rutilans*. We found one specific substance to be produced in these glands, namely the long-chained ester 5-Hexadecenylbutyrate. Potentially, this substance is applied onto a

substrate by extruding the hidden segments of the abdomen. The substance might be spread in a behavior similar to the abdomen-dragging behavior described for polistine wasps making use of the hairbrushes present on the abdominal sternites V and VI. Alternatively, it may play a role during mating. Though the specific function of the substance is not yet completely clear, it is very likely that this substance plays a role in the process of female attraction. At which stage of the mating sequence the substance is involved, though, is not yet known. The fact that large quantities of the substance can be extracted from the abdomens of male *H. rutilans* suggests that the amount of the substance might the result of strong sexual selection. As an alternative, the secretion of the sternal glands may be involved in defense of predators. However, one would expect glands for chemical defense especially in females, because females are probably even more prone to predator attack than males due to their extensive host searching.

Examinations of other chrysidid wasps suggest that sternal glands in association with hairbrushes can only be found in male chrysidid wasps of the Elampine genus *Hedychrum* (see also Lorencova 1973). Apart from *H. rutilans*, the characteristic hairbrushes on the sternites V and VI have been found in *H. nobile*, *H. gerstaeckeri*, and *H. chalybaeum* (J. Kroiss, unpubl. data). In males of other genera as well as in *Hedychrum* females (see also Lorencova 1962), no sternal glands with such an external morphology have been found (J. Kroiss, unpubl. data). At the same time, we found no evidence for pheromones in other chrysidid species than *Hedychrum* species except for males of *Parnopes grandior*, a member of the basal subfamily Parnopini (Octadecanal; J. Kroiss and T. Schmitt, unpubl. data) (Niehuis & Wägele 2004). However, it is not yet clear, where this substance is produced. The absence of hairbrushes on the sternites of the hidden abdominal segments at least makes it unlikely that the substance is also produced in sternal glands. In conclusion, such characteristic sternal glands apparently occur only in males of the genus *Hedychrum*. Preliminary analyses indicate that the substances produced by the different species are very similar if not identical. In *H. gerstaeckeri*, we also found 5-Hexadecenylbutyrate in the sternal glands. The geometry of the double-bond, however, is not yet clear (J. Kroiss, unpubl. data). In *H. nobile* and *H. chalybaeum*, the position of the double-bond of Hexadecenylbutyrate could not be determined. Though not definitively shown, it might be possible that at least some sympatric *Hedychrum* species produce the same substance in their sternal glands. Although the specific function of the substance in the context of mate attraction is not yet known, this might raise a problem: Closely related species are expected to be selected for pre-mating reproductive isolation to minimize the costs of searching for, courting, and mating with partners of other species (Johansson & Jones 2007). Mechanisms to achieve these goals might be character displacement with regard to the pheromone composition (i.e. qualitative differences in the chemical structure or composition; Coyne &

Orr 1997; Frey et al. 1998; Roelofs 1995; Shine et al. 2002; Symonds & Elgar 2004; Symonds & Elgar 2004), active avoidance of heterospecifics (Löfstedt 1990), or seasonal or temporal differences in the timing of pheromone release if the pheromone is involved in remote attraction of mates (Greenfield & Karandinos 1979; Schal 1982). The first and the second scenario do not apply to the *Hedychrum* species under study as they produce the same or at least very similar pheromones and a sensory discrimination seems hardly possible. However, seasonal, temporal, or spatial differences in pheromone release are conceivable, since all species have a specific host-spectrum (Kunz 1994), which might entail such differences in pheromone release.

At today's standard of knowledge, it is not clear whether the sternal glands found in males of the cuckoo wasp *H. rutilans* are homologous to the sternal glands in polistine wasps and in ants. While vespid wasps and ants are phylogenetically closely related, chrysidid wasps are relatively distantly related to these two taxa and the Chrysidoidea branch off rather early in the evolution of aculeate Hymenoptera (Brothers 1999). The evolution of sternal glands early in the evolution of aculeate Hymenoptera would require several losses in other hymenopteran families. Alternatively, sternal glands may have evolved several times in the evolution of aculeate hymenoptera. However, it is hardly possible to decide in favor of one of the alternative hypotheses, since knowledge about sternal glands in other hymenopteran families than the Vespidae, the Formicidae, and now the Chrysididae is largely lacking. Only a broad investigation on the occurrence of sternal glands in other aculeate Hymenoptera families will help to reconstruct the evolution of sternal glands.

Observations of the mating behavior of *H. rutilans* and the involvement of the sternal glands and the hair brushes in the mating sequence, bioassays with *H. rutilans* females, and electrophysiological investigations (GC-EAD) with both sexes of *H. rutilans* will reveal the actual function of the sternal gland secretion of *H. rutilans* males.

12.3 SIGNIFICANCE OF CHCS FOR HOST-PARASITE INTERACTIONS - PLAYING HIDE AND SEEK WITH THE WOLVES

In the second part of this thesis (chapters 7 to 11), we examine the significance of cuticular hydrocarbons (CHCs) for the interspecific communication in host-parasitoid interactions and their evolutionary consequences. First, we report on the relevance of CHCs for the host-location of the cuckoo wasp *H. rutilans* (chapters 7). Hereafter, we demonstrate that the

cuckoo wasps qualitatively mimic the CHCs of their host (chapter 8 and 9) and have an overall reduced amount of CHCs (chapter 10). We propose that these two strategies help the cuckoo wasps to escape detection by their hosts when inside the nest to lay an egg. In chapter 11, we investigate the cuticular ultrastructure of the cuckoo wasp *H. rutilans* particularly with regard to the low CHC level and the specific requirements of the parasitoid lifestyle. Specific issues have already been discussed in the respective chapters. Here, we will generally discuss the evolution of chemical cloaking and deception in *H. rutilans* and other cuckoo wasps and integrate the findings into the knowledge from other taxa. Finally, we discuss the special demands on the cuticle of parasitoid cuckoo wasps and propose the cuticle to be an evolutionary "high-tech" multifunctional structure.

12.3.1 CHCs as kairomones – significance of kairomones for host-finding in parasitoids

In chapter 7 of this thesis, we provide evidence that females of the cuckoo wasp *H. rutilans* use both visual and olfactory cues for the location of host nests. The cuckoo wasps visually orient towards the nest mounds during remote searching and the shape and texture of the nest mounds are used as visual criteria. During close range searching, CHCs of the resident beewolf present in the nest material, help the cuckoo wasp – an "illicit" receiver of the signal – to find and identify host nests. These findings are insofar new to the field of parasitoid host-location that this, together with the results presented in chapter 9, comprises the first report on nest-hydrocarbons (NHCs) in a solitary digger wasp. In addition, the exploitation of NHC for host-finding has so far been reported only rarely (Cervo et al. 1996; Cervo & Turillazzi 1996; Fisher 1983; Fisher et al. 1993). Apart from the identification of the host species, the presence of NHCs in the nest mounds of beewolves might allow the female cuckoo wasps to choose between individual hosts with respect to the individual fit of its own CHCs and the CHCs of its potential host. The cuckoo wasps mimic the composition of the CHCs of their hosts, therefore avoiding countermeasures by their hosts (chapters 8 and 9). However, in *P. triangulum*, we found a dimorphism with respect to the CHC composition (see also Herzner et al. 2007; Strohm et al. 2008) and the CHCs of *H. rutilans* females are much more similar to one of these chemomorphs. This dimorphism is reflected in the beewolf NHCs and cuckoo wasps might be able to optimize their host choice by choosing a host with a CHC profile matching the own CHC composition as well as possible. Optimal host choice with regard to host quality (e.g. size and handling time) has been shown for some parasitoid wasps (Godfray 1994), but the hypothesis of optimal host choice with regard to the

CHC composition of the host by *H. rutilans* females remains to be tested. Multiple choice experiments, both in an olfactometer and in the field, will potentially give new insights, whether female cuckoo wasps choose an especially well-matching host. Additionally, GC-EADs could give information about the physiological sensitivity of cuckoo wasp females for host odors.

In conclusion, the NHCs of female beewolves benefit the illicit receiver of that chemical signal, i.e. searching cuckoo wasp females, but are disadvantageous for the sender, thereby constituting a kairomone.

12.3.2 CHCs and chemical cloaking – how to hide from beewolves

In the chapters 8 and 9, we demonstrate that the cuckoo wasp *H. rutilans* mimics the composition of the CHCs of its host, the European beewolf *P. triangulum*. Bioassays suggest that this chemical mimicry helps the cuckoo wasps to escape detection by their hosts. CHCs are known to be important cues for chemical communication in insects. They have been shown to contain information about species affiliation, sex, and – in social insects – caste affiliation (Singer 1998). Accordingly, many social parasites of social insects, parasitoids, and predators have evolved chemical mimicry or chemical camouflage, i.e. the qualitative imitation of the CHCs of the model species (host or prey species) to evade olfactory detection (Dettner & Liepert 1994; Howard & Blomquist 2005) see chapter 1).

Apart from the qualitative imitation of CHCs, a low amount of CHCs might allow unwanted guests to escape detection. A low level of CHCs might be equivalent to a reduction of recognition cues (Lenoir et al. 2001; Lorenzi et al. 2004) and might serve as a quantitative strategy complementing or being an alternative to chemical mimicry. Such a “chemical insignificance” has been shown in thief ants (Jeral et al. 1997), in slave making ants (D'Ettorre & Errard 1998; Lambardi et al. 2007) and in the social parasite *Polistes atrimandibularis* (Lorenzi & Bagneres 2002).

The data presented in this thesis suggest that the cuckoo wasp uses both chemical mimicry and chemical insignificance to escape olfactory detection by the beewolf females. This is the first example of a parasitoid of a solitary hymenopteran host to employ these two chemical strategies. As outlined before, most examples of chemical mimicry and camouflage deal with parasites, parasitoids, and predators of social insects. Examples from solitary insects are very

rare (but see Geiselhardt et al. 2006). What characteristics make social insects so susceptible to cheating by chemical mimicry in comparison with solitary insects? First, social insect societies store large amounts of resources and they provide a well protected shelter, therefore making them attractive to other species (Hölldobler & Wilson 1990; Seeley 1997). Besides this, they heavily rely on chemicals for social communication (Cardé & Bell 1995; Hölldobler & Wilson 1990) and imitators of these signals – "crackers of the social code" – can gain a great advantage from integrating in the insect society (apart from this, there might be a bias towards the study of social insects as compared to solitary species due to their abundance and their ecological importance). However, even solitary insects might store reasonable amounts of valuable resources and rely on chemical communication, therefore making them prone to chemical mimicry by parasites or parasitoids under certain circumstances.

Some peculiarities of the host-parasitoid system studied in this thesis might have favored the evolution of chemical mimicry and chemical insignificance. First, *H. rutilans* is one of the few cuckoo wasps with a very narrow host-spectrum. *H. rutilans* is a parasitoid of the beewolf species *P. triangulum*, *P. coronatus* (Kunz 1994; Morgan 1984), and *P. venustus* (Linsenmaier 1968). However, in Central Europe *P. venustus* is completely absent and *P. coronatus* occurs only in small refuges (e.g. the "Kaiserstuhl" in southern Germany) and therefore, *H. rutilans* is in most parts of Central Europe effectively monospecific. Additionally, the composition of the CHCs of females of all three *Philanthus* species is rather similar (E. Strohm et al., unpubl. data). This high degree of specialization might facilitate an evolutionary arms race (Davies et al. 1989; Dawkins & Krebs 1979) and the evolution of chemical mimicry. Second, the high level of NHCs present in the nest material (see chapters 7 and 10), might entail a special need for chemical cloaking for parasitoids entering the beewolf nest. An alien invader with a different CHC profile or its traces left during the activities in the nest might be detected very easily by the beewolves. The presence of NHCs is most likely the result of passive transfer from the beewolf cuticle during the extensive digging activities and from the honeybees treated with a special secretion from the PPG of beewolf females (Strohm et al. 2007; Strohm et al. 2008; Strohm et al. in prep). This secretion serves to avoid molding of the bees in the usually warm and humid environment of the beewolf nest (Herzner et al. 2007; Herzner & Strohm 2007; Herzner & Strohm accepted). Both chemical mimicry and chemical insignificance are likely to be adaptive for *H. rutilans* with respect to the high level of NHCs.

Is chemical mimicry expected to be a widespread phenomenon in other chrysidid wasps? We predict the evolution of chemical mimicry predominantly in species with a narrow host-spectrum. Multiple hosts with distinct CHC profiles would make the evolution of chemical mimicry more complicated (but see Bischoff 1934 for a discussion of "host-races" in *Chrysis*

ignita). In Europe, there are only very few cuckoo wasp species, which are specialized on one single or very few host-species apart from *H. rutilans*. For instance, *Parnopes grandior* (Chrysididae, Parnopini) is a specialized parasitoid of the digger wasp *Bembix rostrata* and *Pseudospinolia neglecta* (Chrysididae, Chrysidinae but see Niehuis & Wägele 2004) is a specialized parasitoid of the eumenid wasp *Odynerus spinipes*. Preliminary analyses suggest that both chrysidid species might also employ chemical mimicry (T. Schmitt, J. Kroiss, et al., unpubl. data). Most other species, though, have a broad host-spectrum or knowledge about the host species and their life history is scarce (Kunz 1994).

The incidence and the problems of chemical mimicry in multiple-host parasites have already been investigated. The best studied examples are the larvae of *Maculinea* butterflies, which develop inside *Myrmica* ant nests (Akino et al. 1999; Elmes et al. 2002). It has been demonstrated that the pre-adoption caterpillars of *Maculinea rebeli* possess specific cuticular compounds of both of their host-species, *Myrmica sabuleti* and *Myrmica schencki*, and that they can thereby integrate into both host-species nests (Schlick-Steiner et al. 2004). In *Maculinea alcon*, a social parasite of *Myrmica rubra* and *Myrmica ruginodis* (Als et al. 2001), a mosaic of chemical coevolution (Thompson 2005) has been described (Jackson 2008; Nash et al. 2008): an evolutionary arms race with chemical differentiation between infected populations occurs in *M. alcon* and *M. rubra*, a species with high genetic differentiation between populations ("evolutionary hot-spots"). In contrast, no evolutionary arms race and no chemical differentiation between infected populations have been found in *M. ruginodis*, a panmictic species with high gene-flow ("evolutionary cold-spots"). Alternate hosts might therefore constitute evolutionary refuges for the parasites during periods of evolutionary arms races by one host-species. Thus, chemical mimicry might be possible in multi-host parasites, though the interactions might become much more complicated compared to species specialized on a single host species (see also Brandt et al. 2005).

In comparison with host-species of other cuckoo wasps, the level of NHCs in *P. triangulum* nests might be especially high due to the specific treatment of the prey items (see above). So far, there is no evidence that other wasp species parasitized by cuckoo wasps treat their prey in a similar fashion. Therefore, the NHC level in other species is expected to be much lower compared to *P. triangulum*. Additionally, beewolves construct only one single nest with several brood cells. In the process of nest construction and nest use, NHCs might accumulate only by transfer from the cuticle during digging activities. The NHC level in species, which build an own nest for each brood cell, might therefore be much lower.

In conclusion, the system of *P. triangulum* and the cuckoo wasp *H. rutilans* might be unique and might favor the evolution of chemical mimicry and chemical insignificance. Accordingly,

chemical mimicry is expected to have evolved only in few other cuckoo wasp species. However, chemical insignificance might have evolved also in species with a broad host-spectrum. Chemical insignificance may constitute a more general strategy to escape olfactory detection by the host by providing a reduced strength of recognition cues. It might be even possible to avoid detection or aggression by the hosts using other chemical agents: long-chained alkadienes have recently been suggested to constitute a general cuticular deterrent in a multi-host social parasitic ant (Martin et al. 2007). Potentially, also the absence of methyl-branched alkanes might render discovery of parasites by their hosts ("chemical transparency"; Martin et al. 2008). However, which of these various chemical strategies are used by cuckoo wasps, remains to be tested. Chemical analyses and electrophysiological investigations (e.g. GC-EAD) as well as bioassays will give new insights, which chemical strategies are employed by the different chrysidid species.

12.3.3 Structural colors in the cuckoo wasp *H. rutilans*: is the cuticle a multifunctional structure shaped by several selective pressures?

In chapter 11, we investigate the ultrastructure of the cuticle of the cuckoo wasp *H. rutilans*. We demonstrate by means of SEM-micrographs, reflectance spectral analysis, and theoretical calculations the presence of a cuticular multilayer, which is responsible for the iridescent red coloration. Thus, we have elucidated the proximate cause of the structural coloration in cuckoo wasps. However, the ultimate causes for the coloration are not well understood. In the following, we compile different ultimate functions of structural colors in nature and try to integrate our findings in *H. rutilans* into the current knowledge of biological structural colors.

Colors generated by the interaction of light with periodic nanostructures have been shown to have diverse functions. The most prominent function of these structural colors is the attraction of mates by visual display. In this case, the iridescent coloration is ultimately the result of sexual selection and usually displayed only by males. The best-studied examples of conspecific signaling by structural colors are butterflies (e.g. Berthier et al. 2006; Kinoshita et al. 2002; Potyrailo et al. 2007) and birds (Doucet et al. 2006; Zi et al. 2003). Structural colors play an important role for mate choice and it has been demonstrated in peacocks that females prefer to mate with males with extraordinarily bright and iridescent tail feathers (Loyau et al. 2007). Only recently evidence is accumulating that the expression of these structural colors can be condition-dependent (Kemp & Rutowski 2007; Kemp et al. 2006),

therefore constituting an honest signal for mate choice. For *H. rutilans* and other cuckoo wasps, however, it seems rather unlikely that sexual selection has shaped the evolution of structural colors. Most importantly, both sexes exhibit structural colors and most species do not show sexually dimorphic coloration (except for e.g. *Holopyga fervida*; Kunz 1994). However, we can not exclude that the coloration secondarily plays a role for conspecific messaging.

Several other functions of structural colors apart from sexual signaling have been discussed and demonstrated. For instance, animals might signal toxicity or indigestibility to potential predators using structural colors. Predator deterrence has for instance been suggested for the coloration of the comb-jellyfish *Beroe cucumis* (Ctenophora) (Welch et al. 2006; Welch et al. 2005). This marine invertebrate does not possess any functional eyes and intraspecific visual communication can be excluded as the ultimate reason for color evolution. One of the most impressive examples of structural colors in nature, the golden coloration of the cucurlionid beetle *Pachyrrhynchus congestus pavonius* (Curculionidae) (Welch et al. 2007), has been suggested to be an example of aposematism. These beetles are protected by their hard integument, which renders them inedible for insectivorous birds. Interestingly, it has long been discovered that this species is mimicked by two longicorn beetle species (*Doliops curculionides* and *Doliops geometrica*; Wallace 1879). However, it is not clear, whether the cuticular nanostructures just serve to signal the hardness of the integument, or whether they are contributing to the mechanical properties of the cuticle.

Periodic nanostructures, equivalent to “spaced armor” (Brown & Turner 1985), might have beneficial properties with regard to hardness or stiffness of the cuticle, therefore providing protection against predators (Welch & Vigneron 2007). The mechanical properties of arthropod cuticle have been recently subject to intensive study (Barbakadze et al. 2006; Hillerton et al. 1982; Raabe et al. 2005; Vincent & Wegst 2004), and the micro- and nanoindentation technique (Ebenstein & Pruitt 2006; Enders et al. 2004) might allow elucidating the role of the multilayer for the cuticular mechanical characteristics in cuckoo wasps. In *H. rutilans*, such a protective layer might prevent the sting or the mandibles of the beewolf hosts from penetrating the cuticle. This hypothesis is supported by the fact that basal subfamilies of chrysidid wasps (*Cleptinae, Loboscelidiinae, Amiseginae*), all species being parasites of non-stinging insect larvae, are either completely non-metallic or only partially iridescently colored (Kimsey & Bohart 1990; Kunz 1994). Alternatively, the cuckoo wasps might signal indigestibility due to a hard cuticle to predators like birds analogous to the cucurlionid beetle *Pachyrrhynchus congestus pavonius* (see above). This view is supported by the observation that chrysidid wasps in general show a striking geographic pattern with regard to their coloration: independent from the systematic position, cuckoo wasps within a

certain biogeographic region show a similar coloration pattern (Kimsey & Bohart 1990). This pattern might be the result of the evolution of several distinct Müllerian mimicry-rings (Bates 1862; Joron & Mallet 1998; Mallet & Gilbert 1995; Müller 1879). Sometimes, cuckoo wasps can be mistaken for small iridescent buprestid beetles. Potentially, these two groups mimic each other, but it is unclear which group may be the model and which the mimic.

Apart from their significance in intra- and interspecific communication and their beneficial mechanical properties, periodic cuticular nanostructures might facilitate thermoregulation. This might be of special significance for *H. rutilans*, since the deficiency of CHCs (see chapter 10) might impose the problem of impeded thermoregulation and increased water loss over the cuticle. In addition, cuckoo wasps usually occur in sunny, hot, and dry environments. This lifestyle may require special adaptations (Heinrich 1983). It has been demonstrated that structural colors influence the effect of solar radiation onto the body temperature. Structural colors are able to reflect large proportions of the sunlight and therefore reduce the heating of the insect body. Discoloration (i.e. the loss of iridescent coloration) in high altitude as observed in a lycaenid butterfly sister species pair reduces this strong reflection of light and allows a better thermoregulation in the cold alpine environment (Biro et al. 2003). The same phenomenon has been observed in carabid beetles. The two morphs of *Carabus auronitens escheri* only differ with respect to their coloration. The iridescently bluish-green colored form occurs in altitudes up to 1800 m, whereas the discolored black form can be found in alpine regions of 1900 – 2500 m altitude (Bálint & Makranczy 2007). As in the butterflies, the structural colors are believed to reflect a reasonable part of the solar irradiation and the black morph might be able to heat up more easily in the cold alpine regions. One observation supports the assumption that structural colors might be involved in thermoregulation of chrysidid wasps: One of the very few alpine species, *Chrysis valida*, exhibits a nearly black coloration on the head and the thorax and the iridescent coloration is nearly completely lost (Kunz 1994; Linsenmaier 1959). Structural colors have also been demonstrated to be involved in the thermoregulation of vertebrates, like in the tropical reed frog *Hyperolius viridiflavus*, a species aestivating totally exposed to the sun on dry plants (Kobelt & Linsenmair 1992). All these findings support the hypothesis that structural colors are involved in insect thermoregulation (see also Koon & Crawford 2000; but see Schultz & Hadley 1987).

In *H. rutilans*, the photonic structures might also play a role for thermoregulation. The red reflector described in chapter 11 alone might reduce the impact of solar radiation. This might be an adaptation to the warm and dry habitat, but might also have evolved in response to the reduced amount of CHCs. Additionally the structures found below the exocuticular multilayer might contribute to the reflection of solar irradiation. We found a layered structure with multiple layers with the layer thickness ranging from 550 to 1000 nm. This layered structure

might constitute a second reflector with a reflectance maximum in the infrared range of wavelengths. This structure might contribute to the derivative reflectance maxima observed in the reflectance spectral analysis of *H. rutilans* (see chapter 11). However, only the analysis of IR-reflectance spectra will allow testing for a potentially broad reflectance of the cuticle in the IR range. A broad range of reflected wavelengths can be achieved by three different arrangements of individual layers in a given multilayer (Parker et al. 1998). First, several multilayer stacks with different lattice constants might be superimposed. Second, a single multilayer with a "chaotic" variation of the lattice constant and third, a single multilayer with constantly increasing or decreasing lattice constant might result in a broad reflectance spectrum ("chirped" multilayer; Parker et al. 1998). Broad IR-reflectance has been demonstrated in beetles (e.g. the golden beetle *Anoplognathus parvulus* Parker et al. 1998) and a "chirped" multilayer has been described for the beetle *Aspidomorpha tecta* (Parker et al. 1998). Analogously in *H. rutilans*, the "chirped" cuticular layering observed below the exocuticular multilayer might serve as a broadband multilayer reflector.

In conclusion, the red multilayer reflector and the layered structure below the multilayer reflector observed in *H. rutilans* might have several functions like intra- or interspecific signaling, mechanical protection, and thermoregulation. It has been recognized only recently that this constitutes a very interesting situation, "in which the evolution of a colour-producing structure may by driven by several factors simultaneously, such as a need for mechanical strength, advantageous optical properties, resulting in a multifunctional structure which meets several sets of demands well, but which may or may not be optimal for any of them" (Welch & Vigneron 2007).

As pointed out before, as in *H. rutilans* the ultimate function of some other photonic structures in biology is still not known. This applies for instance to the photonic crystal fiber found in the sea mouse (Aphroditidae, Polychaeta), the only photonic crystal fiber found in nature so far (McPhedran et al. 2001; McPhedran et al. 2003; Parker et al. 2001). However, even if the actual function of a photonic structure is not known, these structures might serve as templates for materials created by humans (i.e. "biomimetics"). This might be either with regard to properties of the materials or its assembly (Chen 2001; Deparis et al. 2008; Deparis et al. 2006; Huang et al. 2006; Large et al. 2007; Lee & Szema 2005; Parker 2002; Parker 2004; Parker & Townley 2007; Sumper & Brunner 2006). Biological photonic structures have been optimized by evolution for over 500 million years (Parker 2000; Parker 2005) and evolution has generated very diverse photonic structures, from simple multilayer reflectors to complex color-switching structures (Rassart et al. 2008; Vigneron et al. 2007). Therefore, man can definitely learn from the evolution of biological photonic structures.

12.4 FINAL CONCLUSIONS

This thesis contributes to the knowledge of the evolution of male sex-pheromones and their potential for mate choice. In the beewolf model system, we show that extraordinarily large amounts of a sex-pheromone are produced in the male postpharyngeal gland. We suggest that the pheromone is an honest signal of male quality and may be used by females to assess with general and specific information about "good genes". Additionally, the pheromone contains information about geographical origin and kinship and might allow mate choice according to an optimal outbreeding model. These results will allow conducting further experiments to elucidate the mate choice criteria in European beewolves. These experiments have the potential to give important insights into the evolution of mate choice in general and mate choice according to chemical signals in particular. Additionally, they might contribute to the knowledge of the evolution of lek mating systems. The description of the newly discovered male-specific substance in *H. rutilans* males adds to the scanty knowledge of insect male sex-pheromones. Bioassays will allow identifying the specific function of this substance.

In the second part of this thesis, we give extensive information about the significance of chemical communication for the interactions of the cuckoo wasp *H. rutilans* and beewolf females. We show that the cuckoo wasps exploit the presence of nest-hydrocarbons for host location and escape detection by the hosts by combined chemical mimicry and chemical insignificance. These results demonstrate the importance of non-cooperative chemical communication in insect host-parasite systems. Future studies will show whether these phenomena are common in other chrysidid wasps or have evolved on account of the peculiarities of the beewolf hosts. We furthermore suggest that the cuticular multilayer evoking the iridescent coloration in cuckoo wasps has evolved in response to several different selective pressures. These results contribute to the expanding field of biophotonics and might stimulate the discussion of multifunctional photonic structures.

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CHAPTER 13

SUMMARY

Communication is an essential process for intra- and interspecific interactions among organisms. It may or may not be advantageous for both the sender and the receiver of the signal. Honest signals may be seen in intraspecific sexual communication as well as in interspecific signals like aposematic coloration. However, deceptive signaling is a common phenomenon and animals may fool predators, prey, or host species as well as conspecifics using deceitful signals.

Communication by means of chemicals is recognized as the oldest and the most widespread form of both intra- and interspecific communication. Especially insects exhibit an incredible diversity of chemical signals. These pheromones serve various functions for intraspecific communication, and sex pheromones play an important role in the context of courtship and mating. While female sex pheromones have been extensively studied in several insect taxa, male sex pheromones have as yet received comparatively little attention despite their significance for honest mate assessment and mate choice. Chemical signals used for interspecific communication are called allelochemicals, and such signals have been described from numerous taxa. Cuticular hydrocarbons, for example, have been shown to be relevant for species recognition and are consequently exploited by (social) parasites to fool the hosts about their actual identity.

This thesis contributes to the knowledge about chemical communication in insects. The first part of the thesis focuses on intraspecific chemical communication and deals with the evolution of male sex pheromones and their potential for female choice. Males of a digger wasp, the European beewolf *Philanthus triangulum* (Hymenoptera, Crabronidae), establish small territories in the vicinity of female nests and apply a sex pheromone from a cephalic gland onto the territory substrate to attract receptive females. The chemical composition of the gland content is characterized using gas chromatography – mass spectrometry (GC-MS): The pheromone comprises a complex blend with (*Z*)-11-eicosenol as the main compound. Apart from (*Z*)-11-eicosenol, we found several other substances with functional groups and an array of hydrocarbons, which can also be found on the cuticle of beewolf males and females.

3D-reconstructions on the basis of NMR imaging data and histological investigations reveal that the pheromone is stored in the postpharyngeal gland (PPG). The PPG is extraordinarily large and makes up about one quarter of the head capsule volume. The large amount of pheromone produced by the males suggests that the amount of pheromone produced is vital for the reproductive success of males and constitutes the result of strong sexual selection. Consequently, quantitative aspects of the pheromone may allow female mate choice according to a "good genes" model. In addition, the composition of the beewolf pheromone is demonstrated to vary qualitatively with, and, thus, to bear information about the geographical origin and kinship of males. This variation might enable females to choose their mate according to a model of "optimal outbreeding". Complementing the investigations of the male sex pheromone, the territorial behavior of beewolf males and the spatial distribution of male territories with respect to female nesting sites is characterized. Spatial statistics reveal that male territories cluster around female nesting sites suggesting that the latter constitute "hotspots" for the formation of male leks. This implies that European beewolves exhibit a "hotspot" lek polygyny as the prevailing mating system. This work provides the basis for a detailed investigation of the criteria for female mate choice, either according to the overall genetic quality ("good genes") or according to the genetic distance of the mate ("optimal outbreeding").

As a contribution to the scarce knowledge of male sex pheromone in insects, a new male-specific substance is described for the cuckoo wasp *Hedychrum rutilans* (Hymenoptera, Chrysididae). The substance is produced in sternal glands and probably serves as a sex pheromone. Morphological investigations suggest that it is applied onto a substrate in a scent-marking process using two characteristic hair brushes. Using GC-MS, the substance has been identified as 5-hexadecenylbutyrate. Future bioassays may reveal the actual function during courtship and mating in *H. rutilans*.

The second part of this thesis deals with the interspecific chemical communication in insects. The significance of cuticular hydrocarbons (CHCs) for the interaction of females of the cuckoo wasp *H. rutilans* and its host, the European beewolf, is investigated. First, it is demonstrated that relatively large amounts of CHCs of beewolf females can be found in the material of female nest mounds. The presence of these nest-hydrocarbons is exploited by cuckoo wasp females as a kairomone for the localization and identification of the host nest. The cuckoo wasps subsequently enter the beewolf nest for oviposition, where they may be attacked by beewolf females. Chemical analyses reveal that cuckoo wasp females mimic the composition of the CHCs of their hosts, and behavioral assays suggest that the cuckoo wasp gains a great advantage from mimicking the composition of the CHCs of its host: the chemical mimicry significantly reduces olfactory detection by the beewolf females and

therefore the incidence of host attacks inside the beewolf nest. Additionally, a possible second mechanism to evade olfactory detection by the beewolves is described: *H. rutilans* females have a drastically reduced amount of CHCs per mm² of the cuticle as compared to their hosts. This low amount of CHCs might be a quantitative means to escape olfactory detection and might represent a "chemical insignificance" strategy. However, the reduced amount of CHCs present on the cuticle might impose the risk of increased water loss on the cuckoo wasp.

The beautifully iridescent cuticle of *H. rutilans* is investigated using Scanning Electron Microscopy, reflectance spectral analysis, and theoretical modeling. The analyses reveal a multilayer reflector in the exocuticle responsible for the coloration. Contributions of the multilayer to secondary functions like thermoregulation and mechanical strength are discussed.

This thesis covers a substantial range of chemical communication in insects. It reveals the structural and functional diversity of chemical signals for both intra- and interspecific communication in two species of Hymenoptera. The data presented here clearly show that chemical signals may be honest like the sex pheromone of male beewolves, or deceitful as it has been shown for the interactions of beewolf and cuckoo wasp females.

CHAPTER 14

ZUSAMMENFASSUNG

Kommunikation ist ein entscheidendes Element intra- und interspezifischer Interaktionen. Sie kann von Vorteil sowohl für den Sender als auch den Empfänger des Signals, aber auch nachteilig für einen der beteiligten Organismen sein. Ehrliche Signale wurden sowohl bei intraspezifischer Kommunikation, z.B. bei sexuellen Signalen, als auch bei interspezifischer Kommunikation, z.B. in Form von aposematischer Färbung, gezeigt. Allerdings sind auch betrügerische Signale ein weit verbreitetes Phänomen, und Tiere täuschen Räuber, Beute, ihre Wirte, oder aber auch Artgenossen mithilfe solcher Signale.

Die Verständigung mit chemischen Botenstoffen ist die älteste und am weitesten verbreitete Form der Kommunikation. Insekten sind für ihre unglaubliche Vielfalt chemischer Signale bekannt. Pheromone dienen der innerartlichen Kommunikation und können die unterschiedlichsten Funktionen annehmen. Sexualpheromone spielen eine große Rolle für die Anlockung von Geschlechtspartnern und für die Partnerwahl. Im Vergleich zu weiblichen Sexualpheromonen haben Sexualpheromone von Männchen bislang wenig Beachtung gefunden, obwohl sie eine große Bedeutung für die Beurteilung potentieller Partner und die adaptive Partnerwahl haben können. Oftmals findet auch eine interspezifische chemische Kommunikation mithilfe chemischer Botenstoffe, so genannter Allelochemikalien, statt. Die für gewöhnlich artspezifischen kutikulären Kohlenwasserstoffe dienen dabei auch der Arterkennung, und werden bisweilen von (Sozial-)Parasiten nachgeahmt, um die Wirte über ihre eigene Identität zu täuschen.

Der erste Teil dieser Arbeit untersucht die intraspezifische chemische Kommunikation bei Insekten. Im Fokus stehen dabei die Evolution männlicher Sexualpheromone und deren Potential für die Partnerwahl. Männchen des Europäischen Bienenwolfs *Philanthus triangulum* (Hymenoptera, Crabronidae), einer Grabwespe, besetzen kleine Reviere in der Nähe von Weibchennestern. Das Substrat dieser Reviere wird mit einem Sexualpheromon aus einer Kopfdrüse markiert, welches der Anlockung von Weibchen dient. Die Analyse der chemischen Zusammensetzung des Pheromons mithilfe von Gas-Chromatographie – Massenspektrometrie (GC-MS) zeigt, dass das Pheromon eine komplexe Mischung verschiedener Substanzen mit (*Z*)-11-Eicosenol als Hauptkomponente darstellt. Neben dieser Hauptkomponente und weiteren Substanzen mit funktionellen Gruppen wurden zahlreiche

Kohlenwasserstoffe nachgewiesen, die auch Bestandteile der kutikulären Kohlenwasserstoffe sind. 3D-Rekonstruktionen auf der Basis von NMR-Tomographiedaten und histologische Untersuchungen zeigen, dass das Pheromon in der Postpharyngealdrüse (PPG), gespeichert wird. Die PPG männlicher Bienenwölfe ist außerordentlich groß und nimmt etwa ein Viertel des Volumens der Kopfkapsel ein. Die große Pheromonmenge, die in der PPG gespeichert wird, legt nahe, dass die Pheromonmenge von entscheidender Bedeutung für den reproduktiven Erfolg der Männchen ist. Dementsprechend ist sie wahrscheinlich die Folge starker sexueller Selektion. Quantitative Aspekte des Bienenwolfpheromons könnten somit eine Partnerwahl im Sinne des „gute Gene"-Modells ermöglichen. Weiterhin zeigt diese Arbeit, dass die Zusammensetzung des Bienenwolfpheromons variabel ist und Informationen über die Herkunft und den Verwandtschaftsgrad der Männchen birgt. Dies könnte es den Weibchen ermöglichen, einen Partner mittleren Verwandtschaftsgrades nach dem Modell des „optimal outbreeding" zu wählen. Über die Analyse des Pheromons hinausgehend charakterisiert diese Arbeit das Territorialverhalten männlicher Bienenwölfe und die räumliche Verteilung der Territorien in Abhängigkeit von der Verteilung der Nester der Weibchen. Die Ergebnisse räumlicher Statistik zeigen, dass Männchenterritorien bevorzugt in der Nähe von Weibchennestern angelegt werden. Die Daten legen nahe, dass die Nistplätze der Weibchen „hotspots" für die Bildung von Leks darstellen. Bienenwölfe weisen demnach eine „hotspot"-Lek-Polygynie als vorherrschendes Paarungssystem auf. Die Ergebnisse der vorliegenden Arbeit erlauben es, zukünftig gezielte Wahlexperimente durchzuführen, die möglicherweise die Kriterien für die Partnerwahl der Weibchen erkennen lassen. Weibchen könnten ihren Partner entweder nach dessen genetischer Qualität („gute Gene") oder anhand seiner genetischen Distanz („optimal outbreeding") wählen.

Diese Arbeit beschreibt weiterhin eine neue männchenspezifische Substanz der Goldwespe *Hedychrum rutilans* (Hymenoptera, Chrysididae). Die Substanz, die wahrscheinlich als Sexualpheromon dient, wird in Sternaldrüsen produziert. Morphologische Untersuchungen legen nahe, dass die Substanz mithilfe zweier Haarbürsten auf ein Substrat aufgetragen wird. Mithilfe der GC-MS Technik wurde die Substanz als 5-Hexadecenylbutyrat identifiziert. Die genaue Funktion der Substanz ist zwar noch nicht bekannt, zukünftige Biotests werden allerdings Aufschluss über die genaue Funktion im Verlauf der Partneranlockung und Paarung von *H. rutilans* geben.

Im zweiten Teil dieser Arbeit wird die interspezifische chemische Kommunikation bei Insekten untersucht. Im Mittelpunkt stehen dabei die parasitoide Goldwespe *H. rutilans* und ihr Wirt, der Europäische Bienenwolf. Untersucht wird dabei die Bedeutung kutikulärer Kohlenwasserstoffe (KKWs) für die Interaktion beider Arten. Zunächst wird gezeigt, dass große Mengen der KKWs weiblicher Bienenwölfe in deren Nesthügeln vorhanden sind. Diese

KKWs werden von weiblichen Goldwespen für die Lokalisation und Identifizierung der Wirtsnester verwendet. Zur Eiablage müssen Goldwespen in das Wirtnest eindringen und können dabei von ihren Wirten angegriffen werden. Chemische Analysen zeigen, dass die Goldwespen die Zusammensetzung der KKWs ihrer Wirte nachahmen. Verhaltensversuche legen nahe, dass die Goldwespen durch diese „chemische Mimikry" im Nest von ihren Wirten weniger gut erkannt werden und folglich auch seltener angegriffen werden. Weiterhin wird ein möglicher zweiter Mechanismus beschrieben, mit dem es den Goldwespen gelingen könnte, einer Erkennung durch ihre Wirte zu entkommen: *H. rutilans* Weibchen weisen im Vergleich zu ihren Wirten eine stark reduzierte Menge an KKWs pro mm² Kutikula auf. Diese geringe Menge an KKWs stellt möglicherweise eine quantitative chemische Strategie dar, mit der die Goldwespen einer Erkennung durch ihre Wirte entgehen können. Für solch eine Reduzierung der KKWs wurde bereits der Begriff „chemical insignificance" vorgeschlagen. Zukünftige Arbeiten werden zeigen, ob diese Reduktion der KKWs das Risiko der Austrocknung der Goldwespen erhöht.

Die metallisch glänzende Kutikula der Goldwespen wird mithilfe von Rasterelektronenmikroskopie, Reflektionsanalysen und theoretischen Modellierungen charakterisiert. Die Untersuchungen zeigen, dass ein Multilayer-Reflektor in der Exokutikula die metallische Färbung hervorruft. Es wird diskutiert, ob dieser Reflektor zu sekundären Funktionen wie der Thermoregulation oder mechanischen Eigenschaften beiträgt.

Diese Arbeit verdeutlicht die strukturelle und funktionelle Diversität chemischer Signale für die intra- und interspezifische Kommunikation zweier Hymenopterenarten. Die Daten zeigen, dass chemische Signale ehrlich oder betrügerisch sein können. Als Beispiel für die ehrliche chemische Kommunikation dient das Sexualpheromon männlicher Bienenwölfe. Beispielhaft für betrügerische chemische Kommunikation werden die Interaktionen der Goldwespe *H. rutilans* mit ihrem Wirt, dem Europäischen Bienenwolf, betrachtet.

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ERKLÄRUNG

laut § 6, Abs. 1, Nr. 3 der Ordnung zum Erwerb des akademischen Grades eines Doktors der Naturwissenschaften (Dr. rer. nat.) an der Universität Regensburg

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.
Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Regensburg, den 29.09.2008